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Delayed phytotoxicity syndrome in Louisiana rice caused by the use of thiobencarb herbicide

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**DELAYED PHYTOTOXICITY SYNDROME IN LOUISIANA RICE CAUSED
BY THE USE OF THIOBENCARB HERBICIDE**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by

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Table of Contents

Acknowledgements	ii
Abstract	vi
Chapter 1	Literature Review and Research Objectives 1
	1.1 Behavior of Thiobencarb in the Environment 2
	1.2 Reductive Dechlorination of Thiobencarb Caused Delayed Phytotoxicity Syndrome in Rice 5
	1.3 Current Understanding of Reductive Dehalogenation in Other Chlorinated Aromatic Compounds 14
	1.4 Rice Soil Redox Conditions and DPS..... 22
	1.5 Problems and Perspectives..... 24
	1.6 List of Objectives 26
Chapter 2	Phytotoxicity of Thiobencarb and Dechlorinated Thiobencarb to Rice as Determined by <i>in vitro</i> and Greenhouse Bioassays 28
	2.1 Introduction 28
	2.2 Materials and Methods 31
	2.3 Results 38
	2.4 Discussion 59
Chapter 3	Relationship between Reductive Dechlorination of Thiobencarb and Redox Potential in a Soil Column 64
	3.1 Introduction 64
	3.2 Materials and Methods 68
	3.3 Results 75
	3.4 Discussion 87
Chapter 4	Microorganisms Implicated in the Dechlorination of Thiobencarb in South Louisiana Rice Field Soil 91
	4.1 Introduction 91
	4.2 Materials and Methods 94
	4.3 Results 102
	4.4 Discussion 118
Chapter 5	Summary and Future Research Needed 126
	5.1 Summary 126
	5.2 Future Research Needed 128

References	131
Vita	139

Abstract

Thiobencarb (TB), widely used for the control of broadleaf weeds, grasses, and sedges in rice fields, is considered safe for rice plants when used at recommended rates. TB's reductive dechlorination product, dechlorinated thiobencarb (DTB), is highly toxic to rice. TB is naturally transformed into DTB in field soils in certain areas in Japan and the United States. The resultant syndrome is called delayed phytotoxicity syndrome (DPS). This research was conducted to characterize DPS in Louisiana, to compare the toxicity of TB and DTB to rice, to determine uptake and retention rates of TB and DTB by rice, to confirm that soil microorganisms convert TB to DTB, to determine factors affecting the dechlorination of TB, and to develop methods for isolating dechlorinating microorganisms.

An *in vitro* bioassay developed in this study showed that seedling heights were reduced as concentrations of TB and DTB in soil increased. The effective dosage for 50% reduction in height, using Lafitte rice, was 6.6 µg/ml for TB and 0.3 µg/ml for DTB. By developing and using a gas chromatography/mass spectrometry (GC/MS) method, it was shown that DTB was not taken up preferentially by rice plants. Rice plants absorbed and accumulated more TB than DTB when exposed at equal concentrations. The toxic effects of TB and DTB to rice seedlings was additive. When rice cultivars were evaluated for sensitivity to DTB, M201 was more tolerant than Bengal, Cocodrie, and Lafitte. The conditions affecting the transformation of TB into DTB in soil were studied using a special apparatus developed to measure the redox potential of soil columns at different depths. Reductive dechlorination of TB peaked after 14 days incubation, at a position in the soil column corresponding to an Eh of -230

mV. TB was converted to DTB *in vitro* in a conducive soil, but not after the soil was autoclaved. Bacterial and fungal isolates from conducive soil inoculated into sterile soil suspensions, or the soil column, all failed to dechlorinate TB. Repeated attempts to isolate the organisms responsible for dechlorination of TB in Louisiana rice field soils failed.

Chapter 1

Literature Review and Research Objectives

Thiobencarb (TB) (formerly named benthocarb) is the common name for S-[(4-chlorophenyl) methyl]-diethylcarbamothioate (Ahrens et al. 1994). Trade names currently used for this herbicide include Bolero, Abolish, and Saturn (Ashton & Monaco 1991; Mabury et al. 1996). TB has been widely used in Japan on rice since 1970. By 1974, the treated area reached more than 50% of the total rice area. In the United States, TB was first registered for use on rice in 1982, and was re-registered in 1997 (EPA, 1997). Although used primarily on rice (95%), it is also used on lettuce, celery, and endives (in Florida). Approximately 550,000 kg are applied annually in the U. S. on 188,260 ha (186,234 ha of which is rice) (EPA, 1997). About 11% of the rice in Arkansas, Louisiana, California, and Texas is treated with TB annually. The average rate of TB used on rice was 3.2 kg ai/ha. The two formulations of TB currently being used on rice are an 84% emulsifiable concentrate (8EC) and a 10% granular (10G).

TB is widely used for the control of broadleaf weeds, grasses, and sedges, including barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) and sprangletop (*Leptochloa fascicularis* (Lam.) Gray) (Ahrens et al. 1994). TB belongs to the carbamothioate family, which includes butylate, cycloate, diallate, EPTC, molinate, pebulate, triallate, and vernolate. They are derivatives of carbamic acid with one of the oxygen atoms replaced by a sulfur atom, or with other substitutions (Casida et al. 1974).

TB is a systemic, pre-emergence herbicide that acts by inhibiting the growth of shoots of emerging seedlings (Ahrens et al. 1994). The biochemical basis for the biological activity of TB is not well known. It is probably involved with inhibition of the biosynthesis of fatty acids and lipids, proteins, isoprenoids, flavonoids, and porphyrin

(Ahrens et al. 1994; Prakash et al. 1990). Other hypotheses have been proposed, including the conjugation of acetyl coenzyme A and other sulfhydryl-containing biomolecules by thiocarbamate sulfoxides, which may be the active form of some of the thiocarbamate herbicides (Casida et al. 1974). Rice seedlings absorb TB rapidly through the mesocotyl, coleoptiles, roots, and the first leaf (Ahrens et al. 1994). Animals and plants normally metabolize TB, with residual amounts in the plants being very small (Ishikawa, 1980).

1.1 Behavior of Thiobencarb in the Environment

Mabury et al. (1996) reviewed the environmental fate of TB in California rice fields. TB tended to be redistributed among the major environmental compartments after application. These compartments include atmosphere, water, soil and sediment, and plants and animals. The physical properties of TB play an important role in determining the redistribution of TB in the environment. Pure TB is a pale yellow liquid with a boiling point of 126-129 C. It is readily soluble in most organic solvents and slightly soluble in water (approximately 30 µg/ml at 20 C). TB is considered marginally volatile with a value of 2.7×10^{-7} of Henry's law constant, which relates the vapor pressure to the maximum water solubility (Mabury et al. 1996). The equilibrium distribution of TB between sediment and water is represented by the organic carbon coefficient, K_{oc} , which supposes the soil to be entirely composed of carbon from the soil organic matter. With a K_{oc} of 1380, TB is considered intermediate between weak binding (e.g., MCPA [(4-chloro-2-methylphenoxy) acetic acid] with a value of 112 K_{oc}) and strong binding (e.g., DDT (dichlorodiphenyltrichloroethane) with 243,000 K_{oc}). TB can become concentrated into plants and animals in rice fields. This is believed to be proportional to the solvent

partition coefficient (K_{ow}). TB is the most readily bioconcentrated among the major rice pesticides used in California. The values of K_{ow} for bensulfuron methyl, carbofuran, MCPA, methyl parathion, molinate and TB were 4, 95, 272, 933, 223, and 2630, respectively (Mabury et al. 1996). On the other hand, TB is biodegradable. Microbial hydrolysis and oxidation in sediment and water are generally the most important determinant of its fate in the field (Ishikawa et al. 1976). Since TB is applied at times of great light intensity, sunlight is very important in its degradation. TB does not absorb appreciable UV energy itself, but is very susceptible to attack by aqueous oxidants occurring naturally in sunlit rice field water (Mabury et al. 1996). Thus, TB is generally non-persistent in water.

After being applied to rice fields, TB is more likely to be found in the soil than in the floodwater (Ishikawa et al. 1976, 1977; Ross & Sava 1986). The partition of TB associated with soil was approximately 10 times more when applied pre-flooded to soil than when applied to standing water, as TB has more time to bind to soil prior to flooding. As a result, sensitized aqueous photolysis is more significant as a dissipation route when TB is applied to water than when it is applied to dry soil. TB also is stable to degradation by hydrolysis and is stable under anaerobic aquatic conditions, with a calculated half-life in sediment of 5.4 years. TB is degraded moderately slowly under aerobic conditions with calculated half-lives of 27-58 days in a typical rice soil (Nakamura et al. 1977). TB slowly mineralizes in soil without forming significant quantities of non-volatile degradates. Bound residues and CO_2 were the primary products from soil metabolism studies, occurring in proportions of 23-42% and 42-47%, respectively. Aqueous residues did not exceed 4.5% in soil metabolism studies. TB was

moderately mobile to essentially immobile in the tested soils. Mobility generally decreased with increasing clay content, organic matter content, and cation exchange capacity (EPA 1997).

The behavior of TB in the rice field environment can be illustrated by the following example. A study was conducted to examine the fate of TB in a California rice field (Ross & Sava 1986). The granular form of TB was applied by fixed wing-aircraft before rice seedlings emerged above the water surface (1-3 leaf growth stage). TB was applied at 4.48 kg/ha, and the water was held 6 days at a depth of 26 cm. After the 6-day holding period, water depth was maintained at 17 cm with intermittent inflow and outflow. The maximum concentration in air was $1.4 \mu\text{g}/\text{M}^3$ on day 1. Changes in TB concentrations in water, soil, and plant are illustrated in Figure 1-1. In the water, TB concentration was only $0.08 \mu\text{g}/\text{g}$ on the first day, but quickly reached $0.57 \mu\text{g}/\text{g}$ 2 days after application. The TB concentration in the water remained relatively constant for 2 to 8 days after application, but dropped rapidly to $0.056 \mu\text{g}/\text{g}$ at 16 days after application, and to $0.008 \mu\text{g}/\text{g}$ (8ppb) at 32 days after application. In the soil, TB concentrations were relatively constant during the 32 days of observation. On the day of application, TB reached $3.25 \mu\text{g}/\text{g}$ in the soil, peaking at 6 days after application to $3.86 \mu\text{g}/\text{g}$, and then dropped to $2.02 \mu\text{g}/\text{g}$ at 8 days after application due to the outflow and inflow of water. TB concentration in the soil still remained $2.33 \mu\text{g}/\text{g}$ 32 days after application. In the plants, TB concentrations remained low between the first day and 2 days after application, but peaked at $1.75 \mu\text{g}/\text{g}$ 4 days after application. TB concentrations in the vegetation remained high until 16 days after application when it dropped to $0.8 \mu\text{g}/\text{g}$. Thirty-two days after application TB concentration dropped to $0.17 \mu\text{g}/\text{g}$. This study provided very

important information regarding the behavior of TB in rice field environments. However, a concern with this study was that the standard deviations were relatively high for soil samplings. This probably resulted from inadequate sample procedures as noted by the authors themselves (Ross & Sava 1986).

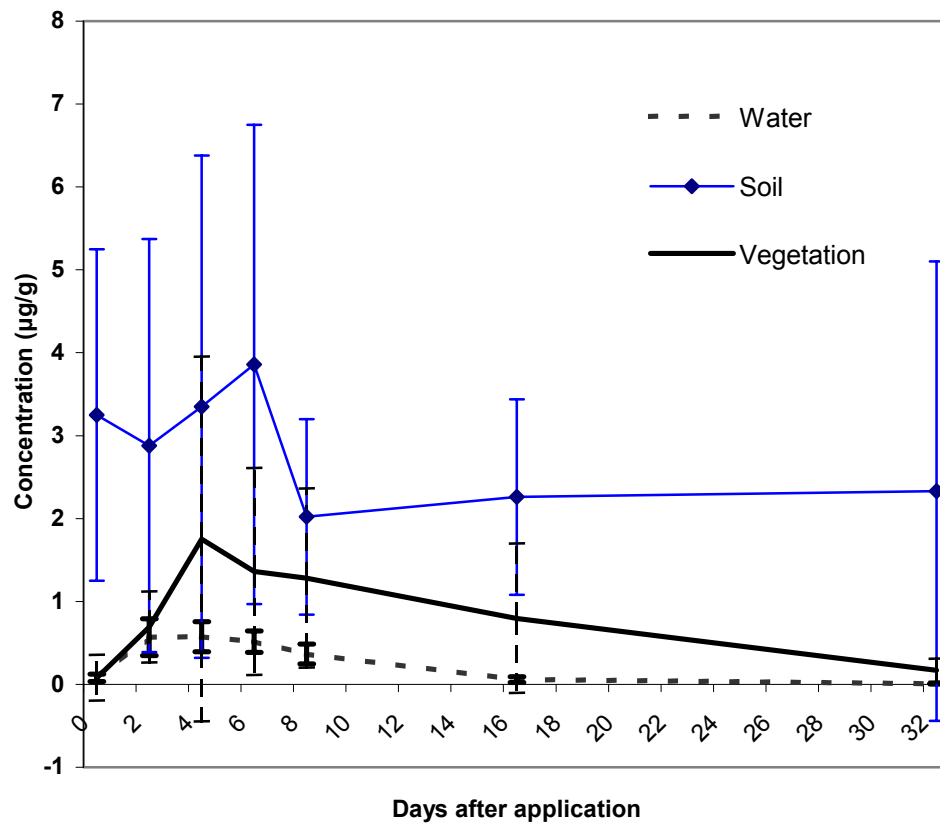


Figure 1-1. Thiobencarb concentrations in water, soil, and vegetation over time after application into a California rice field (Plotted from the data of Ross and Sava 1986). Error bars represent standard deviations with $n = 16$.

1.2 Reductive Dechlorination of Thiobencarb Causes Delayed Phytotoxicity Syndrome in Rice

1.2.1 The Problem.

In 1976, a disorder with serious “dwarfing” of rice was first noticed in Japan (Ishikawa et al. 1980). The disorder was associated with rice fields that had been treated with TB. Soil samples from these fields were analyzed and a reductive dechlorination

product of TB, dechlorinated TB (DTB), was found to be present in significant amounts only at locations showing dwarfing symptoms. DTB had not been shown in previous studies of TB degradation in soils, animals, plants, and by light (Ishikawa et al. 1980). A similar disorder was identified in Louisiana in 1991 (Bollich et al. 1996; Groth & Sanders 1996; Groth et al. 1999) and in California in the late 1990s (Tjeerdema & Crosby 2000). The phytotoxicity symptoms included stunting, excessive tillering, curvature or "fishhooking" of tillers, overall brittleness of the plant (Groth & Sanders, 1996) and delay of maturity (Bollich et al. 1996). The above disorders were called delayed phytotoxicity syndrome (DPS). Contrary to reports from Japan where affected acreage remained constant (Moon and Kuwatsuka 1984), DPS in Louisiana rice fields appeared to be spreading, with the affected acreage increasing to 20,000 acres in 1993 (Groth et al. 1999). In addition to reports related to TB, DPS has been associated with several other rice herbicides including quinclorac, triclopyr, propanil and 2,4-D (Groth et al. 1999).

A biological cause of DPS was suggested because damaged plants were in irregularly shaped areas and unevenly distributed within fields (Groth et al. 1999). Autoclaving the soil prevented the dechlorination of TB. Dechlorination appeared proportional to the ratio of conducive/non-conducive soil in the mixtures (Groth and Sanders 1996), and repeated application of TB shortened the lag period for dechlorination (Tatsuyama et al. 1981; Moon & Kuwatsuka 1984, 1985a). Furthermore, dechlorination was inhibited by application of certain antibiotics (Moon & Kuwatsuka 1985a) or fungicides (Groth et al. 1999). Dechlorination of TB peaked at the temperature range of 25-30 C and at pH 7 (Moon & Kuwatsuka 1985b). These are the ideal conditions for growth of many microorganisms.

1.2.2 Phytotoxicity Studies

The disorder of DPS necessitates the comparison of phytotoxicity to rice caused by TB and DTB. However, while there are fairly extensive studies on the phytotoxicity of TB (Manun and Shimizu 1978a, 1978b, 1979a, 1979b), there is only one preliminary report currently available on the phytotoxicity of DTB (Tatsuyama et al. 1981). Thus, a brief review on the phytotoxicity of TB may help to shed light on that of DTB. TB has a high inter-genus selectivity between rice, a C₃ plant, and barnyardgrass, a C₄ plant (Prakash et al. 1990). The primary site of action of thiocarbamate herbicides was found to be located in either the shoot or leaf tissue. Differences in stomatal behavior, amount of epicuticular wax and silica contents between C₃ and C₄ plants were considered to be the factors responsible for differential selectivity of thiocarbamate herbicides (Prakash et al. 1990). TB is usually nontoxic to rice plants at the recommended rate of 4.5 kg ai/ha. Other plant species vary in their sensitivity to TB. Many grasses appear to be exceptionally sensitive to TB. The most sensitive species was ryegrass for which the EC₂₅ based on mortality (LC₂₅) was 0.021 kg ai/ha. The most sensitive dicot was lettuce, with an EC₂₅ based on mortality estimated to be 0.302 kg ai/ha (EPA, 1997). The basis for the selective toxicity is still not completely understood. The absorption and translocation of TB were rapid for both rice plants and several weed species (Nakamura et al. 1974). However, the translocation was more rapid and extensive in barnyardgrass compared to rice plants. In another study, Ishikawa (1980) showed that rice and barnyardgrass had similar metabolic pathways, but different degradation rates, with rice degrading TB faster than barnyardgrass.

Understanding the phytotoxicity of DTB and possibly screening for DTB-tolerant rice cultivars would be of practical use, as using these cultivars would minimize the phytotoxicity from exposing plants to DTB in TB-dechlorinating rice fields. Rice cultivars have shown differential reactions toward TB (Uno et. al. 1977). Whether there are also different sensitivities among available rice cultivars toward DTB is unknown.

In a preliminary study that tested the phytotoxicity of DTB to rice, Tatsuyama et al. (1981) used pre-germinated rice seeds grown in centrifuge tubes with soil incorporated with TB and DTB. They found that rice seedling height was significantly reduced at 5 µg/ml (dry soil basis) of DTB while TB only inhibited seedling height at concentrations of 40 µg/ml or higher. At 2.5 µg/ml of DTB, seedling heights were similar to those exposed to 40 µg/ml of TB. However, the quantitative relationship between the concentration of DTB and rice height was not studied.

1.2.3 Factors Affecting Dechlorination of TB

Tatsuyama et al.(1981) reported DTB was produced under flooded conditions, but not under dry conditions. When soils from DPS fields were incorporated with TB and incubated under flooded conditions, the concentrations of DTB increased from less than 0.2 µg/ml at 1 wk to 4.9 µg/ml after 5 wk of incubation, while the concentrations of TB fell from 19.8 µg/ml to 4.8 µg/g. Laboratory, greenhouse and field studies indicated DPS only occurs under prolonged flooded conditions in rice fields (Tatsuyama et al. 1981). Draining DPS fields alleviates the severity of symptoms (Groth & Sanders 1996). Given the complexity of the soil environment, the dechlorination of TB can be directly or indirectly affected by many factors.

1.2.3.1 Soil Characteristics

Moon and Kuwatsuka (1984) tested 17 different soils, including nine different rice soils, for their ability to dechlorinate TB under flooded conditions (Moon & Kuwatsuka, 1984). Among these 17 soils, only two had TB-dechlorinating activity, even after they were amended with additional organic matter (0.5% rice straw powder). The chemical and physical compositions of these soils were compared. The conducive soil (Ohshiro soil) had higher available phosphate content, lower phosphate absorption coefficient and lower free iron oxide. When only paddy soils were compared, the exchangeable Ca and K, clay %, total N, and CEC were lower in the conducive soils than in nonconductive soils. Other properties, such as pH, total C, and C/N ratio were not distinguishable between the conducive and nonconductive soils.

Although some soils may not dechlorinate TB directly, they can support dechlorination once inoculated with TB-dechlorinating suspensions. Sixteen soils were flooded and then inoculated with a TB-dechlorinating soil to see if the soils would support dechlorination (Moon & Kuwatsuka 1985a). During the 30-day study, five soils had no dechlorinating activity, two soils had little activity, and nine soils had high dechlorinating activity. Interestingly, some upland soils supported dechlorination after inoculation with TB-dechlorinating suspensions under flooded conditions. Of the properties studied, the available phosphate content had a high positive correlation with dechlorinating activity, while concentrations of C and N had highly negative correlations. After 2 weeks of incubation, the pH values in all soils ranged from 6.8 to 7.4, and redox potential values (Eh) ranged from -200 to -250 mV. However, there was no relationship

between TB-dechlorinating activity and the changes in pH and Eh found during incubation (Moon & Kuwatsuka 1984)

1.2.3.2 Nutritional Factors

In Japan, the dechlorination of TB required the amendment of soil with a certain amount of organic matter (Moon & Kuwatsuka 1984, 1985b). Amendment with 0.1% of glucose, sucrose, galactose, lactose, mannitol or ascorbate enhanced dechlorination. Dechlorination was not supported without organic amendment, or with too much organic amendment. Without amendment, TB was degraded gradually, but DTB was not formed. In the case of too much amendment (e.g., 1% glucose), TB remained relatively constant over a period of 50 days, while DTB was not formed. This behavior was very similar to the result when the soil had been autoclaved (Moon & Kuwatsuka, 1984).

Media also affected the dechlorination of TB. Ten different bacterial media were inoculated with 1/10,000-diluted suspension of the TB-dechlorinating soil (Moon & Kuwatsuka 1987a). The dechlorination of TB only occurred in the following four media: 1) medium prepared with a soil extract of TB-dechlorinating soil (Oshiro soil); 2) a mineral salt (MS) medium with a diverse salt composition supplemented with 0.1% yeast extract; 3) MS with 0.4% Casamino acid; 4) and MS with 0.05% starch. The dechlorination rate was faster in the first two media, with lag periods of 5 days. The lag period for the others was 14 days. Bacterial media, which contained the reducing agent cysteine (at 0.3 g/L), failed to support the dechlorination of TB. The same was true of mineral salt medium with ammonia salt, regardless of which organic amendments were used (Moon & Kuwatsuka 1984).

Mineral salt composition affected TB-dechlorination. Some non-supportive soil extracts became supportive of dechlorination after being supplemented with certain mineral salts. In supportive soil extracts, the dechlorination activities were affected by the addition of certain mineral salts or nitrogen sources. In a supportive (Oshiro) soil extract, 0.07% or 0.14% of glycine, or 0.09% KNO₃ promoted the dechlorination of TB (Moon & Kuwatsuka 1987a). When the above chemicals were added at higher rates, they inhibited dechlorination. A non-supportive extract became supportive of dechlorination of TB after appropriate amounts of glucose, FeCl₃, and AlCl₃ were added to the extract (Moon & Kuwatsuka 1985b).

In the MS, vitamins alone did not support dechlorination, even after inoculation with an activated suspension. However, by combining vitamins and amino acids, or by adding 0.1% yeast extract, this mineral salt medium became supportive for dechlorination.

1.2.3.3 Environmental Condition, Redox Potential, and pH Factors

Tatsuyama et al. (1981) measured redox potentials of a diluted soil suspension in a test tube after 2, 4 and 6 weeks of incubation. The treatments with added organic matter showed very low redox potentials after 4 weeks of incubation. The redox potentials ranged from -290 mV (with 0.4% rice straw powder), to -330 mV (with 0.5% soluble starch), while the control (no additional organic matter) showed +115 mV. Treatments with less than 0.4% rice straw powder did not show significant amounts of DTB formation. They had a much higher redox potential (-10mV) than the 0.5% soluble starch (-315 mV) after 2 weeks incubation. It appeared that reducing conditions needed to be maintained for a certain period of time for dechlorination of TB to occur.

Reductive conditions are not only required for dechlorination of TB, but are also required for DTB persistence in soil. DTB was added to the soil to see if seedling height was inhibited with different levels of organic matter (Tatsuyama et al. 1981). The DTB only inhibited seedling growth when high amounts of organic matter were applied, which encouraged anaerobic conditions. However, the specific concentrations of DTB were not measured. Thus, this study was only a preliminary study and was not conclusive.

Pre-incubation of soil suspension under aerobic conditions for 1 week appeared to be required for dechlorination. However, the depth of water used for the pre-incubation did not have a consistent effect on dechlorination of TB, as dechlorination occurred when soil was incubated under 0.3, 1, 3, and 6 cm of water (Moon & Kuwatsuka 1984).

Adding certain weak reductants into the soil or soil suspension expedited the development of reductive conditions. Suspensions with 2.5% activated conducive soil added had shorter lag periods for dechlorination when 0.1% ascorbate or 0.1% starch was added. However, thioglycolate, sodium sulfide, sodium hydrosulfite, and ferrous sulfate did not increase dechlorinating activity when they were added without additional organic matter (Moon & Kuwatsuka 1985a). When coenzymes were added, after the suspension was amended with 0.1% starch, 0.02% NADPH increased the dechlorination rate faster than 0.02% of ADP or ATP during an 11 to 12 day observation period (Moon & Kuwatsuka 1987a).

1.2.3.4 Microbial Factors

Determining which microbes are involved in DPS will contribute to the prevention or the control of DPS. No organisms have been isolated and proven to be able to

dechlorinate TB under a defined system. Two groups of soil microbes have been implicated in the dechlorination of TB: 1) Gram-positive, facultative anaerobic bacteria were suggested as the cause of DPS in Japan (Moon & Kuwatsuka 1987a,); 2) a group of fungi were implicated in DPS in south Louisiana (Groth & Sanders 1996; Sanders et al. 1996).

Evidence for the microbial nature of reductive dechlorination of TB came from early studies. In a flooded Oshiro soil, the dechlorination of TB did not occur until 20 days after the first application of TB (Moon & Kuwatsuka, 1985a). Dechlorination took only 10 days after the second application of TB. Dechlorination was even faster during the third application, taking only a few days. Evidence that the TB-dechlorinating organisms were enriched after application of TB came from the following test. TB-dechlorinating suspension was sampled at two stages: when TB was being actively dechlorinated (most active stage) and 4 days after the complete disappearance of TB. Suspensions from these two stages were then used as inocula to test the dechlorinating rate in phosphate buffer (pH 7) or in water. The rate of dechlorination of TB was highest when the most active stage of dechlorination was used than when the later (less active) stages were used, especially when they were inoculated into a buffer where pH had been adjusted to 7.

1.2.3.5 Inhibitors of DPS

Moon & Kuwatsuka (1985c) found that methoxyphenone and BNA-80 (a phenyl methyl sulfonate derivative) were highly effective for inhibition of microbial mediated dechlorination. In South Louisiana, it was discovered that certain fungicides, such as iprodione, could prevent DPS (Sanders et al. 1996).

Certain antibiotics affect the dechlorination of TB in active TB-dechlorinating suspensions. Among the antibiotics tested, bacitracin, penicillin, streptomycin, gentamycin, tetracycline and chloramphenicol inhibited dechlorination while colistin A slightly inhibited dechlorination during an 11 to 12 day period (Moon & Kuwatsuka, 1985a). Cycloheximide, an antibiotic that inhibits fungi, did not inhibit the dechlorination of TB at 100 µg/ml in the active TB-dechlorinating soil suspension.

1.2.3.6 Other Factors

In Japan, dechlorination in an active TB-dechlorinating soil appeared to be specific, as only certain types of chlorinated compounds were dechlorinated. P-Cl-phenyl and m-Cl-phenyl, N,N-dimethyl-, N,N-dipropyl-, and dithio derivatives of TB were dechlorinated after 12 to 14 days of incubation. However, o- Cl-phenyl, chlorpropham, chlornitrofen, r-BHC, and p,p-DDT were not dechlorinated (Moon & Kuwatsuka, 1985b).

1.3 Current Understanding of Reductive Dehalogenation in Other Chlorinated Aromatic Compounds

Halogenated aromatic compounds are important pollutants because they often enter the environment in substantial quantities, are toxic, resist degradation, and accumulate in sediments and biota (Holliger et al. 1999). Halogenated aromatic compounds are generally resistant to biodegradation because the halogen atom is both larger and more electron withdrawing than hydrogen. As a result, these halogens can have detrimental steric and electronic effects upon the ability of enzymes to catalyze the required degradation reactions (Crooks & Copley 1993). The direct dechlorination of chlorinate-substituted aromatic compounds can be achieved either through direct oxidative dechlorination, which is catalyzed by extremely nonspecific microbial dioxygenases from

Pseudomonas, *Alcaligenes*, and *Moraxella* species (Fetzner & Lingens 1994), or through reductive dechlorination, which is favored by anaerobic conditions (Bouchard et al. 1996; Suflita et al. 1982; Utkin et al. 1994).

In direct oxidative dechlorination, molecular oxygen is introduced into the aromatic ring and produces an ortho-dihydroxylated product and halogen ions. Recently, reductive dechlorination, which replaces a chlorine atom with a hydrogen atom, has been reported for many of the chlorinated pesticides, as well as, other environmental pollutants (Quensen et al. 1990; Suflita et al. 1982). Several anaerobic bacteria have been isolated from enriched sediment or sludge, including *Desulfomonile tiedjei* DCB-1 (DeWeerd et al. 1990), *Desulfitobacterium dehalogenans* (Utkin et al. 1994), *Desulfitobacterium hafniense* (Christiansen & Ahring 1996), *Desulfitobacterium chlororespirans* (Sanford et al. 1996) and *Desulfitobacterium frappieri* (Bouchard et al. 1996). These anaerobic bacteria were able to use formate or pyruvate as the electron donor and chlorinated aromatic compounds as electron acceptors. Energy was conserved, probably through electron transport phosphorylation (Dolfing & Harrison 1992; Mackiewicz & Wiegel 1998; Suflita et al. 1982).

Several bacterial cultures, both mixed and pure, have been identified which are capable of reductive dechlorination of chlorinated aliphatic and aromatic compounds. Although numerous reports and reviews on microbial reductive dechlorination activities are available, this process is still not completely understood (Holliger et al. 1999). Many studies involved co-metabolic or abiotic reductive dechlorination, or dechlorination by mixed cultures, facultative anaerobes or aerobic bacteria. It should be noted that several aerobes could catalyze reductive dechlorination reactions. In some aerobes, this reaction

appeared to be dependent on glutathione, while a requirement for NADH as an electron donor was observed for others (Mohn & Tiedje 1992). The compounds reductively dechlorinated by these organisms were usually intermediates formed during the aerobic degradation of chlorinated aromatics (Holliger et al. 1999).

Reductive dechlorination under strictly anoxic conditions was observed for a variety of anaerobes, including methanogenic and homoacetogenic bacteria (Madsen & Licht 1992; Mohn & Tiedje 1992; Holliger et al. 1999). The involvement of methanogenic bacteria in reductive dehalogenation was suggested by the observation that the dehalogenation was completely inhibited by 2-bromoethanesulfate, a specific inhibitor of methanogenic bacteria. However, some questions have been raised recently about the direct involvement of methanogenic bacteria (Löffler et al. 1997). In most cases, the process appeared to be mediated in cell-free systems by heat-stable compounds at low velocities. Due to the dechlorinating activity of tetrapyrrole-cofactors, such as corrinoids, iron porphyrins, and coenzyme F430 in aqueous solutions, it has been proposed that, in many dechlorinating bacteria, the dechlorination reaction is a co-metabolic activity of enzymes containing these co-factors as prosthetic groups. For some co-metabolically dechlorinating bacteria, it has been demonstrated that tetrapyrrole-containing enzymes are indeed responsible for the dechlorination reaction they catalyze. This type of reductive dechlorination reaction is extensively described (Fetzner 1998).

In terms of microbial metabolism, the halogenated compounds can be transformed by pure strains under anaerobic conditions by three major routes:

1.3.1 Co-metabolic Reductive Dehalogenation

In a co-metabolic process, dechlorination is not coupled to growth. It is a form of gratuitous metabolism carried out by enzymes or cofactors, which normally catalyze other reactions. The microorganisms involved have no apparent benefit from a co-metabolic transformation. Many methanogenic, acetogenic, sulfate-reducing and iron-reducing bacteria perform co-metabolic dehalogenation with no benefit to the organism. Co-metabolic activities have been found mainly with haloalkanes (Fetzner 1998). The reduction of tetrachloromethane by *E. coli* K12 under fumarate-respiring conditions and by the denitrifying *Pseudomonas sp.* strain KC, as well as the dechlorination of tetrachloromethane by *Shewanella putrefaciens* 200 presumably are co-metabolic processes that are mediated by electron carriers of the respiratory electron transport chains. Free Co(I) corrinoids and iron (II) porphyrins have been found to catalyze the reductive dechlorination of arylhalides, haloalkanes, and haloalkenes (Gantzer and Wackett 1991). Compared with metabolic dehalogenation, co-metabolic dechlorination processes proceed at much lower rates (Fetzner 1998).

Gantzer and Wackett (1991) showed that transition-metal co-enzymes (Vitamin B12, coenzyme F430, and hematin) were capable of catalyzing reductive dechlorination of chlorobenzenes and polychlorinated ethylene. All of these enzyme systems contain redox-active metal centers, and are usually referred to as transition-metal co-factors. In anaerobic bacteria, transition-metal cofactors have a biological role as electron carriers. Since electron transfer by these transition-metal co-factors is not very specific, a wide range of halogenated aromatic compounds can be transformed, usually yielding reductive dechlorination.

1.3.2. Reductive Dehalogenation Linked to Carbon Metabolism

Reductive dehalogenation reactions are not restricted to strictly anaerobic bacteria (Apajalahti & Salkinoja-Salonen 1987). The purple nonsulfur bacteria *Rhodospirillum rubrum*, *R. photometricum* and *Rhodopseudomonas palustris* grow phototrophically on C2 and C3 halocarboxylic acids in the absence of oxygen (reviewed by Fetzner 1998). Reductive removal of the halogen substituent (s) is followed by assimilation of the corresponding carboxylic acids. A new enzymatic function was discovered during growth of *S. chlorohydroquinone*, dehalogenase (PcpC), which shows slight similarity to some enzymes of the θ class of the glutathione S-transferase super-family. The enzyme catalyzes the reductive dechlorination of tetrachloro-p-hydroquinone first to tri- and then to 2,6-dichlorohydroquinone. Cys-13 of tetrachlorohydroquinone dehalogenase was found to form a covalent adduct with glutathione. A glutathione-dependent reductive dehalogenase also is involved in the degradation of γ -hexachlorocyclohexane by *S. paucimobilis* UT26, catalyzing the reductive dechlorination of 2,5-dichlorohydroquinone to chlorohydroquinone and then to hydroquinone.

1.3.3 Reductive Dehalogenation as a Respiratory Process

Reductive dehalogenation as a respiration process was termed “dehalorespiration” (Holliger et al. 1994, 1999). Dehalorespiration represents a new type of metabolism by anaerobic and facultative anaerobic bacteria. The dehalogenation rates in dehalorespiration are believed to be much higher than those of co-metabolism, as the organisms benefit from the process and are quickly enriched (Mohn & Tiedje 1991). A sulfate reducing bacterium, *Desulfomonile tiedjei*, was the first organism isolated in pure culture and shown to be capable of obtaining energy from the dechlorination of 3-

chlorobenzoate into benzoate (DeWeerd et al. 1990; Mohn & Tiedje 1992). In its dehalorespiration, *D. tiedjei* used 3-chlorobenzoate as an electron acceptor and hydrogen gas or formate as the electron donor. Several other bacteria were later isolated and showed to be capable of dehalorespiration. Many of these bacteria belong to the genus *Desulfitobacterium*, including *D. dehalogenans* (Utkin et al. 1994), *D. halfniense* (Christiansen et al. 1996), *D. chlororespirans* (Löffler et al. 1996), *D. frappieri* (Bouchard et al. 1996), and an unidentified species PCE-1 (Gerritse et al. 1996). Interestingly, an unidentified 2CP-1 strain, which is close to myxobacterium and is a facultative anaerobic bacterium, was isolated and capable of using 2-chlorophenol and 2,6-chlorophenol as electron acceptors (Cole et al. 1994). This bacterium was able to grow under aerobic conditions, but dechlorination only occurred under anaerobic conditions. The characteristics of isolated bacteria capable of reductive dechlorination of halogenated aromatic compounds are summarized in Table 1-1.

The enzymes that catalyze the reductive dechlorination of halogenated aromatic compounds were studied (Ni et al. 1995; Löffler et al. 1996). Three different reductive dehalogenases capable of dechlorinating halogenated aromatic compounds were purified and their properties are summarized in Table 1-2.

1.3.4 Reductive Dechlorination in Fungi.

Reductive dechlorination is not restricted to bacteria. Recently the white rot fungus, *Phanerochaete chrysosporium*, was found capable of reductive dechlorination during the mineralization of 2,4,6-trichlorophenol compounds (Reddy et al. 1998). The fungus is

Table 1-1. Characteristics of bacteria capable of reductive dechlorination of halogenated aromatic compounds^{x, z}.

	<i>Desulfitobacterium</i> spp.						Others		
	<i>D. chlororespirans</i>	<i>D. dehalogenans</i>	<i>D. frappieri</i>	<i>D. halfniense</i>	<i>D. sp. strain PCE1</i>	<i>Desulfomonile tiedjei</i>	<i>Desulfovibrio dechloroacetivorans</i>	Strain	
Morphology	Curved rods	straight or curved rods	rod shaped	slightly curved rod	helical	straight rods	Short curved rod	2CP-1 rods	
Mobility	+	-/+	-	++	+	-	+	nd	
Gram	-	+	+	+/-	+	-	-	-	
G+C (mol%)	Nd	45.0	46	47.0	nd	49.0	nd	nd	
pH range (opt)	6.8-7.5	6-9 (7.5)	6.0-9.0 (7.5)	7	6.2-8.8 (7.2)	6.5-7.8 (6.8-7.0)	nd	7.5	
Temperature (opt)	15-37 (37)	13-45 (38)	15-45 (38)	22-45 (35-37)	19-42 (34-37)	30-38 (37)	(30)	25	
Spore forming	+	-	+	+	-	-	nd	nd	
Pyruvate fermentation	Acetate	lactate + acetate	+	acetate + CO ₂	acetate + CO ₂	lactate + acetate	nd	-	
Electron acceptor (halogenated compound)	2,3-DCP 2,4,6-TCP 3-Cl-4-OHPA	3-Cl-4-OHPA 2, 4-DCP	nd	3-Cl-4-OHPA	PCE, 2-CP 2,4,6-TCP 3-Cl-4-OHPA	3-CB PCP	2-CP 2,6-DCP	2-CP 2,6-DCP	
Electron donors	H ₂ , formate pyruvate	H ₂ , formate pyruvate	pyruvate	pyruvate, tryptophan	formate pyruvate	H ₂ , formate pyruvate	acetate, fumarate lactate propionate pyruvate, ethanol	acetate	
Reference	Sanford et al. 1996	Utkin et al. 1994	Bouchard et al. 1996	Christiansen and Ahring 1996	Gerritse et al. 1996	DeWeerd et al. 1990	Sun et al. 2000	Cole et al. 1994	

^x Abbreviations: 3-Cl-4-OHPA: 3-chloro-4-hydroxyphenylacetate, 2-CP: 2-chlorophenol. 2, 4-DCP: 2,4-dichlorophenol, 3-CB: 3-chlorobenzene, PCP: pentachlorophenol.

^z Modified from Fantroussi et al. 1998.

Table 1-2. Properties of reductive dehalogenases capable of dechlorinating halogenated aromatic compounds^z.

Enzyme	3-Cl-benzoate reductive dehalogenase	Cl-OHPA reductive dehalogenase	Cl-OHPA reductive dehalogenase
Organism	<i>Desulfomonile tiedjei</i>	<i>Desulfitobacterium dehalogenans</i>	<i>Desulfitobacterium hafniense</i>
Substrate	3-Cl-benzoate	Cl-OHPA	Cl-OHPA
Localization	Membrane	Membrane	Membrane
Molecular mass (kDa)	64 (α); 37 (β)	48	47
Apparent Km (μ M)	ND	20	ND
Specific activity in cell extract (nkat/mg)	2.5	2.5	9.1
Specific activity of the purified enzyme (nkat/mg)	300	167	103
Cofactors	Heme Fe/S	Corrinoid Fe/S	1 corrinoid 12 Fe, 13 S
Oxygen sensitivity t _{1/2} (min)	Stable	90	100

^z Abbreviations: Cl-OHPA, 3-chloro-4-hydroxy-phenylacetate; ND, not determined.

Modified from Holliger et al. 1999.

well known for degrading lignin and cellulose and for the degradation of some chlorinated aromatic compounds (Yadav et al. 1995). Two extracellular heme peroxidases, lignin peroxidase (Lip) and manganese peroxidase (MnP), as well as a H₂O₂-generating system, apparently constitute the major extracellular components of this organism's lignin degradative system (Aust 1990; Reddy et al. 1998). The multistep pathway is initiated by a Lip- or MnP-catalyzed oxidative dechlorination reaction to produce 2,6-dichloro-1,4-benzoquinone. The quinone is reduced to 2,6-dichloro-1,4-hydroxybenzene, which is reductively dechlorinated to yield 2-chloro-1,4-trihydroxybenzene. The product may undergo further reductive dechlorination to yield 1,4-trihydroxybenzene. In this pathway, the chlorine at C-4 is oxidatively dechlorinated,

whereas, the other chlorines are removed by reductive process in which chlorine is replaced by hydrogen.

The discovery of the reductive dechlorination pathway in the white mold fungus may provide supporting evidence for the argument that fungi are responsible for the dechlorination of TB. However, many questions remain to be solved. One question would be the paradox of the reductive condition (required for the dechlorination and buildup of the DTB) and the aerobic habitat for fungal growth. One possibility is that the TB was being dechlorinated at the aerobic or near aerobic condition and then migrated into the reductive zone for persistence. This does not seem likely, since TB has little mobility in the soil (Mabury et al. 1996) (the mobility of DTB in soil is unknown). Another problem is that fungi must exist in the transition zone, in which the dechlorination occurs when the soil is aerobic and becoming anaerobic, or the enzyme must remain active after the fungi become inactive. The enzymes produced by fungi would have to penetrate into the reductive zone to reductively dechlorinate TB.

1.4 Rice Soil Redox Conditions and DPS

One of the most important practices in rice production is to flood the rice field to control weed growth. Flooded soils are characterized by a steep gradient of oxygen between oxic surface soil and anoxic bulk soil, and between anoxic bulk soil and rhizospheric soil of submerged or partially submerged plants (Khalid et al. 1977; Patrick & Delaune 1972; Patrick et al. 1985; Reddy et al. 1980). Because of limited O₂ penetration, a flooded soil can be differentiated into a surface oxidized layer and an underlying reduced layer. The major factors determining the thickness of the oxidized surface layer include the supply of O₂ at the soil surface and its consumption rate in the

soil. Redox potential can be very useful in characterizing the two layers existing at the surface of flooded soil. Soil redox potential measurements are most useful in flooded soils, which are characterized by spatial and temporal variations with respect to the availability of substrates, oxygen, and alternative electron acceptors. As a consequence of the different physicochemical conditions, microbial communities within each of these compartments are different (Conrad 1996; Gambrell & Patrick 1978; Ludemann et al. 2000). Water covering the soil or filling soil pores prevents the entry of gaseous oxygen and decreases oxygen diffusion by a factor of more than 10,000. When a soil is flooded, the oxygen level begins to decline and drops to 0 within about a day (Patrick et al. 1985).

The pH of most soils tends to change toward the neutral point after flooding. The equilibrium pH for waterlogged soil is usually between 6.5 and 7.5 (Connell & Patrick 1968; Patrick et al. 1985). Flooding the soil also causes an increase in the concentration of ions in the soil solution. The physiochemical measurement that best differentiates a flooded soil from a well-drained soil is the oxidation-reduction, or redox potential. Well-aerated soils are characterized by Eh values equal to or higher than +400 mV, whereas waterlogged soils may have values as low as -300 mV if the reduction processes are sufficiently intense. The redox potential of a soil is determined by the degree of oxidation or reduction of the various redox systems in the soil. These include the oxygen, nitrate, manganese, iron, and sulfur systems, as well as various organic compounds (Patrick et al. 1985). Free oxygen functions both chemically and biologically to maintain these systems in the oxidized form. The redox potential of a flooded soil gives a fairly good indication of the intensity of oxidation or reduction in the soil. The redox potential of a submerged soil is a mixed potential from a number of oxidation-

reduction systems and is difficult to interpret in terms of any single system. A change in the potential signals a shift in the equilibrium and usually involves changes in more than one system.

1.5 Problems and Perspectives

Although DPS has been known for over 20 years, many problems remain:

1. DTB has only been detected from soil, no attempt has been made to extract DTB from rice plants showing DPS symptoms;
2. The phytotoxicity of DTB has not been compared and contrasted with TB and no attempt to explore the toxicity mechanism of DTB has been made;
3. The difference in tolerance of rice cultivars to DTB has not been studied;
4. The relationship between reductive dechlorination of TB and soil and environmental conditions has not been studied in a soil system;
5. Microorganisms responsible for the dechlorination of TB have not been isolated and confirmed in a system where only the tested organisms were maintained.

1.5.1 Gas Chromatography/Mass Spectrometry System to Detect TB and DTB.

Since TB and DTB are very similar in their chemical characteristics, thin layer chromatography was not able to separate them (Ishikawa et al. 1980). Previous studies of TB and DTB used Gas Chromatography equipped with a NP detector to detect TB and DTB concentrations (Moon & Kuwatsuka 1984). While this method was adequate for detection of samples where the amount of sulfur is present in trace quantities, it would not be specific for soil samples where S is present in large quantity and laborious procedures would be needed to reduce the interference (Yamada 1982). Gas chromatography/mass spectrometry (GC/MS) has become a very popular tool in

detecting chemicals such as pesticides in soils. The approach is to separate components of a mixture using gas chromatography and identify them based on their characteristic mass spectra. One advantage of GC/MS is its ability to use characteristic ions of a compound to detect it in a mixture. This procedure is termed selected ion monitoring (SIM) mode. In our research, a comparison of mass spectra of TB and DTB gave TB five characteristic fragment peaks, (m/e) 44, 72, 100, 125, and 257 while DTB gave characteristic fragments of 44, 72, 100, 91, and 223 (Chen et al. 2000a). These characteristic fragment peaks offer promising ways to detect TB and DTB using GC/MS with SIM mode. Although detection of TB from water samples using GC/MS has been reported (Hada et al. 2000), the same procedure was not used in the detection of DTB.

1.5.2 Stratification of Redox Conditions in Flooded Rice Soil and the Reductive Dechlorination of TB

Because of limited O₂ penetration into the soil, a flooded soil can be differentiated into a surface oxidized layer and an underlying reduced layer. Redox potential, which is a measure of the electron availability or potential, can be very useful in characterizing the two layers existing at the surface of flooded soil (Patrick et al. 1996). A platinum (Pt) electrode readily transfers electrons either to or from the medium, but presumably does not react chemically with the medium. When a Pt electrode is coupled with a suitable half cell of known potential, reducing systems tend to transfer electrons to the electrode while oxidizing systems tend to take electrons from the electrode. The redox potential corrected with hydrogen (Eh) is the more common expression of soil redox potential since it is readily determined with a platinum electrode. Along with pH, redox potential (Eh) defines conditions under which important biogeochemical processes take place (Patrick et al. 1996).

1.5.3 Isolation of TB-dechlorinating organism(s)

The organism(s) responsible for dechlorination of TB in rice field soil remains to be isolated. Preliminary results showed that the dechlorinating activity of TB in a TB-dechlorinating suspension had been enriched as shown by the shortening of the lag period for the second and the third application of TB (Moon & Kuwatsuka 1984). However, these results were inconclusive, as all the applications of TB were added into the same suspension, and no control was included to test if the incubation process itself could have changed the conditions of the suspensions to make it more conducive. Furthermore, it may be possible that enzymes, which catalyze the reductive dechlorination of TB, might have been induced through the repeated application of TB, rather than the dechlorinating organisms being enriched. If the organisms were enriched through repeated application of TB, the dechlorination of TB likely involved dehalorespiration, as the DTB was not further degraded. If the dechlorinating organisms were not enriched from the dechlorination process, it may indicate co-metabolic dechlorination. Further studies are needed to address this question.

1.6 List of Objectives

The overall objective of this research was to characterize DPS as it occurs in Louisiana as to conditions favoring development of the disorder, comparison of toxicity of TB and DTB, comparison of uptake and retention of TB and DTB, confirmation that microorganisms convert TB to DTB, and development of methods that promote isolation of dechlorinating microorganisms. Specific objectives of the research include:

1. To quantify the phytotoxic effects of TB, DTB and mixtures of TB and DTB on rice using *in vitro* and greenhouse bioassays.

2. To determine if there are different reactions to DPS among selected rice cultivars, to determine the potential for screening for tolerance to the disorder.
3. To develop a procedure to efficiently identify and quantify TB and DTB concentrations in rice plants, soil/soil suspensions, and water.
4. To develop an *in vitro* model system to assay for dechlorination of TB by microorganisms under controlled conditions.
5. To develop an apparatus to accurately and efficiently measure the redox potential at different depths in a soil suspension/soil column, and to use this apparatus to determine the relationship between redox potential and the reductive dechlorination of TB.
6. To use the above apparatus to identify the most likely area to isolate dechlorinating microorganisms and to assay isolated microorganisms for their ability to dechlorinate TB.
7. To identify microorganisms isolated from conducive rice field soils that can dechlorinate TB, using greenhouse tests and the soil column assay.

Chapter 2

Phytotoxicity of Thiobencarb and Dechlorinated Thiobencarb to Rice as Determined by *in vitro* and Greenhouse Bioassays

2.1 Introduction

Thiobencarb (TB) is a systemic, pre-emergence herbicide that acts by inhibiting shoot growth of emerging seedlings. TB is more effective for controlling weeds of Graminaceae and Cyperaceae families than broadleaf plants (Nakamura et al. 1974). Within the Graminaceae, rice plants are tolerant to TB at the recommended rate of 4.5 kg. a.i./ha while other plants, especially barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.), are very sensitive to TB in early growth stages after germination. The basis for the selective toxicity is still not completely understood. By exposing seedlings of rice and barnyardgrass to ¹⁴C-thiobencarb and then detecting the distribution of radioactivity, Nakamura et al. (1974) reported that the absorption and translocation of TB were rapid in roots, leaves, or seeds of rice and four weed species. However, the translocation was more rapid and extensive in barnyardgrass when compared with rice. This difference was more significant in seedlings than in grown plants. In another study, Ishikawa (1980) reported that rice and barnyardgrass had similar metabolic pathways, but different degradation rates. Rice degraded TB faster than barnyardgrass.

Phytotoxicity associated with the use of TB was first noticed in Japan in 1976 (Ishikawa et al. 1980). A reductive dechlorination product of TB, dechlorinated thiobencarb (DTB), was found in significant amounts in soils when rice was showing dwarfing symptoms. A similar disorder was found in southwest Louisiana in 1991 (Bollich et al. 1996; Groth & Sanders 1996; Groth et al. 1999) and in California in the late 1990s (Tjeerdema & Crosby 2000). The phytotoxicity symptoms caused by DTB

included stunting, excessive tillering, curvature or "fishhooking" of tillers, and an overall brittleness of the plant (Groth & Sanders 1996). These symptoms did not appear until approximately 2 weeks after the application of thiobencarb. Symptoms expressed on rice after exposure to DTB were collectively called delayed phytotoxicity syndrome (DPS). Contrary to the disorders observed in Japan, DPS in Louisiana appeared to be spreading rapidly. The affected acreage had increased to 8,094 ha by 1993. In addition to TB, DPS has been associated with several other rice herbicides, including quinclorac, triclopyr, propanil, and 2,4-D (Groth et al. 1999).

Herbicide bioassays have been used to estimate and compare the dose-response curves for many herbicides, or mixtures of herbicides, and the findings were then related to the basic mechanism of phytotoxicity (Streibig et al. 1993). Bioassays of DTB and TB would be of particular interest as they differ only in one chlorine atom while showing significant differences in phytotoxicity to rice. Differential tolerance to TB has been reported between indica and japonica rices (Unno et al. 1977). However, the relative phytotoxicity of TB and DTB to rice has not been compared. The only report was a preliminary study by Tatsuyama et al. (1981), who reported that DTB was much more toxic to rice seedlings than TB. Rice seedlings exposed to 2.5 $\mu\text{g/g}$ DTB showed DPS symptoms similar to rice seedling exposed to 40 $\mu\text{g/g}$ of TB. However, the highest TB concentration (80 $\mu\text{g/g}$) did not consistently inhibit rice seedling growth, thus this was an incomplete study.

Since TB and DTB are very similar in their chemical characteristics, thin layer chromatography techniques were not able to separate them (Ishikawa et al. 1980). Previous studies of TB and DTB used gas chromatography equipped with a NP detector

to detect TB and DTB concentrations (Moon & Kuwatsuka 1984). While this method was adequate for detection of samples where the amount of S, P, or N were present in trace quantities, it was not specific for soil samples where S was present in large quantities, and laborious procedures had to be used to reduce the interference (Yamada 1982). Gas chromatography/mass spectrometry (GC/MS) has become a popular tool for detecting chemicals, such as pesticides in soils. This method is used to separate components of a mixture and identify them based on their characteristic mass spectra. One advantage of GC/MS is that it uses characteristic ions of a compound to detect the material in a mixture. This procedure is termed selected ion monitoring (SIM) mode. A comparison of mass spectra of TB and DTB showed that TB had five characteristic fragment peaks, (m/e) 44, 72, 100, 125, and 257 while DTB had characteristic fragments of 44, 72, 100, 91, and 223 (Chen et al. 2000a). Although detection of TB in water samples using GC/MS has been reported (Hada et al. 2000), a GC/MS method for the detection of DTB in plant tissue and soil samples has not been described.

The objectives of this study were: 1) to quantify the phytotoxic effects of TB, DTB and mixtures of TB and DTB on rice using *in vitro* bioassays, and to determine if there are differential reactions among selected rice cultivars; 2) to study the phytotoxic effects of TB and DTB using bioassays conducted in a nonconductive soil in the greenhouse; 3) to study the phytotoxic effects of TB and its dechlorinated product DTB in a conducive soil in greenhouse tests; 4) to develop a GC/MS procedure to quantify TB and DTB concentrations in rice plants, soil and water; and 5) to compare the persistence of TB and DTB in rice plants and attempt to relate persistence to phytotoxicity of TB and DTB. Preliminary results from this study were reported earlier (Chen et al. 2000b).

2.2 Materials and Methods

2.2.1 Chemicals. Technical grade TB (96.7%) was provided by Valent's Dublin Laboratory (Dublin, CA). DTB (98.9%) was provided by Kumiai Chemical Industry C., Ltd., Tokyo, Japan. Unless indicated otherwise, the other chemicals used in this study were obtained from Difco Laboratories, Detroit, MI.

2.2.2 *In vitro* Test

2.2.2.1 Stock solutions of TB or DTB. The stock solutions of TB and DTB were made by dissolving 0.2 g of each chemical into 20 ml 95% ethanol to obtain approximately 10,000 µg/ml stock solutions. A series of dilutions were then made with ethanol. Concentrations of each chemical were obtained by adding the appropriate amount of stock solution into liquefied medium and then mixed well (Table 2-1). For example, 5 µl of 10,000 µg/ml stock solution of TB was added to 50 ml medium to obtain 1µg/ml final concentration.

2.2.2.2 Rice seeds. Seeds of four rice cultivars Bengal, Cocodrie, Lafitte, and M201 were used. The seeds were harvested before they were fully matured, thus reducing field contamination by microorganisms. The dry seeds were dehulled and then surface-disinfected with 50% Clorox bleach (active ingredient: 5.25% sodium hypochlorite, the Clorox Company, Oakland, CA) at room temperature for 30 min before washing with autoclaved, distilled water and then transferring onto potato dextrose agar (PDA) plates. After incubation at 28 C for 4-5 days, sterile, germinated seeds were selected and used for the *in vitro* phytotoxicity test described below.

Table 2-1. The incorporation of TB or DTB from stock solutions into MS agar (MA) in Magenta boxes

Final concentration (µg/ml)	Volume of MA medium (ml)	Concentration of stock solution (µg/ml) ^Z	Stock solution added (ml)
100	30	100,000	0.03
10	30	10,000	0.03
1	30	1,000	0.03
0.1	30	100	0.03
0.01	30	10	0.03
0.001	30	1	0.03
CK	30	Ethanol	0.03

^Z The stock solution of 100,000 µg/ml TB or DTB was obtained by weighting 1 g of TB or DTB and then ethanol was added to a total volume of 10 ml. The stock solutions of lower concentrations were obtained by a series of 10X dilution, e.g. the stock solution of 10,000 µg/ml was obtained by adding 1 ml of 100,000 µg/ml stock solution into 9 ml of ethanol.

2.2.2.3 Magenta box *in vitro* test. Agar medium was prepared by mixing agar (8 g/L) with Murashige-Skoog salts (4.3 g/L) (Sigma M5524, Sigma Chemical Co., St. Louis, Mo). TB and DTB were incorporated into the medium at concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 µg/ml (final concentration), respectively. An equal volume of acetone (0.02 ml in 30 ml media) was used as blank control. A sterile, germinated seed was transferred into an individual Magenta box, which contained 30 ml MS agar (MA) incorporated at the above concentrations of TB and DTB. The seeds were incubated at 23 to 25C under 12/12 h alternative light/dark conditions using fluorescent light. After 24 days, the height of each rice seedling was measured. There were five replications for each treatment. The experiment was conducted twice.

2.2.2.4 Phytotoxicity of TB, DTB and their mixtures on rice. The experiment was arranged as a factorial with TB and DTB as two factors. Each factor had six levels: 0, 1, 2, 5, 10, 20 µg/ml (final concentration) for TB, and 0, 0.1, 0.2, 0.5, 1, 4 µg/ml (final concentration) for DTB. The MS medium contained MS salts and agar. The media were prepared by dissolving MS salts into distilled water at 4.3 g/L. This solution was then distributed into 200 ml-flasks at 100 ml/flask. Bacto Agar (Difco, Becton Dickinson Co., Sparks, MD) at 7 g/L was then added into each flask. The flasks were autoclaved at 15 psi for 20 min and then stored at 54 C in a water bath. TB or DTB, prepared as a series of stock solutions containing 10,000x of final concentrations, was added to the medium in each flask at 10 µl/100 ml medium, and then the flask was vortexed, and 2.5 ml of medium was dispensed into sterile test tubes (1.5x15 cm) topped with aluminum foil.

A sterile, germinated seed (cv. Lafitte) prepared as described in the previous section was transferred into each tube and the resulting plant was incubated in an

illuminated incubator (818 Low Temperature Illuminated Incubator, Precision Scientific Inc.), which was set for 28 C with 12 /12 h alternative light and dark conditions. The seedling heights were measured at 2-3 day intervals for approximately 20 days. There were 40 replicates for each treatment. The experiment was conducted twice.

2.2.2.5 Tolerance of rice cultivars to TB and DTB. To evaluate the tolerance of rice cultivars to TB and DTB, four rice cultivars, Bengal, Cocodrie, Lafitte and M201, were selected. Each cultivar was evaluated at two levels of TB (8 and 40 µg/g) and two levels of DTB (0.2 and 1 µg/g). The preparation of medium, transfer of germinated rice seeds and incubation conditions were the same as previously described, except that TB was incorporated into the medium using 97% technical grade TB rather than from the 10,000 stock solution.

The experiment was conducted twice. In the first experiment, Bengal, Cocodrie, and Lafitte each had 8 replications while M201 had 16 replications. In the second experiment only Cocodrie, Lafitte, and M201 were tested with 23, 16, and 24 replications, respectively.

2.2.3 Greenhouse bioassays. A non-conductive soil (field soil in which TB is not naturally converted to DTB) was chosen to avoid the complicating factor of natural microbial transformation of TB to DTB. A Crowley silt loam soil with pH 5.7-6.1 and 0.88-0.92% organic matter was collected from the top 15 cm at the Rice Research Station in Crowley, LA. To test the effect of autoclaving soil on the phytotoxicity of TB and DTB, half of the soil was autoclaved (15 psi, 60 min) on 2 consecutive days while half of soil was not autoclaved. Two hundred grams of soil was placed into each 473-ml Styrofoam cup (Dart Container Corp., Mason, MI 48854) and flooded with tap water at

full level for 14 days. The soil/water in each cup was stirred with a wooden stake before the application of TB or DTB. One gram of TB or DTB was first dissolved in acetone, and then a series of 10x dilutions in acetone was prepared. From each dilution, 0.2 ml was applied into each cup to obtain TB or DTB concentrations of 100, 10, 1, 0.1 and 0.01 $\mu\text{g/g}$ (dry soil basis). A control with 0.2 ml of acetone was included. There were five replicates for each treatment. After the TB or DTB was applied to the soil in each cup, the soil was stirred again. A one-week-old Bengal rice seedling was then transferred into each cup. The water level was maintained to keep the cup full at all times. The test was conducted in January of 1999. The greenhouse temperature ranged from 18-25 C. After 2 weeks, the plants were harvested and plant heights, total fresh weight, and root fresh weight were recorded. Rice plant height, total fresh weight, shoot fresh weight, and root fresh weight were converted into percentage of inhibition by comparing each data set with the means from the control. The study was repeated.

2.2.4 Phytotoxicity of DTB transformed from TB in conducive soil. Soil and rice tissue samples in the greenhouse study with conducive soil were used for quantification of TB and DTB concentrations, which were then correlated to plant height, fresh weight and tiller numbers. This study was conducted from March 2000 through April 2001 in the greenhouse. The design of the experiment was a randomized complete block design with four replications. There were 11 treatments in each block, including nontreated, TB only, a white mold fungus (ATCC34540) plus TB, and each of eight unidentified bacterial isolates (isolated from DPS-conductive soil by Dr. D. E. Groth of Rice Research Station, Crowley, LA), plus TB. In previous studies, the bacterial and fungal isolates did not cause any visual injury to rice plants when inoculated individually. A total of 1.8 kg

of Crowley silt loam soil conducive to DPS was placed into autoclavable bags, and autoclaved for 1 h. The bags were stored at 23-25 C for 24 h and autoclaved for 1 h a second time. Each bag of soil was then transferred into a plastic pot (18-cm-diameter). The top of the bag was opened and filled with tap water. Fungal inocula were produced on a sterile medium consisting of autoclaved rice hull: rice grain (50:50) and then incubated at 28 C for 1 week. Bacterial inocula were produced by inoculating nutrient broth and incubating at 28 C for 2 day. Each container received 10 ml of fungal or bacterial inoculum. TB was applied as Bolero 8 EC at 4.5 kg ai/ha based on the area of the top of the pots. Bengal rice seedlings, pre-germinated and grown in sterilized soil in a tray for 7-10 days, were transferred into each pot. Pots were kept full of tap water. Plant heights and tiller numbers were checked at 41 days after transplanting (DAT), then water was drained from each pot, the rice plants were carefully removed with roots still attached, washed with tap water to remove soil particles, and then dried by blotting with paper towels. Total fresh weight was obtained by weighing rice plants and roots from each pot. Then roots were separated from leaves and culms and stored in a freezer for extraction of TB and DTB. Soil and water remaining in the pot were mixed with a wooden stake until a suspension was formed. Approximately 50 g of suspension was collected for each pot and stored at -20 C for extraction of TB and DTB. The experiment was conducted twice.

2.2.5 Persistence of TB and DTB in rice plants. Intact three-leaf Bengal rice seedlings were collected from the greenhouse where they were grown in a sand/soil/peat mixture (1:2:1), washed thoroughly with tap water, and dried by blotting with paper towels. A 15-g seedling sample was placed into a 200-ml flask containing 100 ml aqueous solution of

TB and DTB (each at 1 µg/g), with roots and a small portion of the culm of each seedling immersed in the solutions. Water was used as a control. The seedlings were incubated under fluorescent light at 23 to 25 C for 24 h. The seedlings were then removed from the flasks and washed in running water for 5 min. A 5-ml sample of solution was collected in each flask before and immediately after the incubation. The rice seedling roots were then submerged in 100 ml of distilled water in 200-ml flasks for an additional 4 days. Water inside each flask was replaced with fresh tap water twice a day. The seedlings were then removed from the flasks, and washed with tap water. The roots were then dipped into acetone for 30 s to remove surface TB and DTB. The seedlings were then separated into root and shoot samples by cutting with scissors. All plant tissues were frozen at –20 C until extraction. Each treatment was repeated three times, and the experiment was conducted twice.

2.2.6 Extraction of TB and DTB. The extraction method for TB and DTB from root samples and leaf/culm samples followed the procedure described by Ross and Sava (1986). The extraction of TB and DTB from soil and suspension samples was by the procedure described by Moon and Kuwatsuka (1984).

2.2.7 Quantification of TB and DTB in plants and soils and their phytotoxicity to rice plants in conducive soil. The quantification of TB and DTB concentrations in soils and plants was carried out through the use of GC/MS analysis. Hexane extracts of soil suspensions and dichloromethane (DCM) extracts of plant tissues were analyzed using this method. GC/MS was run on a Hewlett Packard GC 6890 Gas Chromatograph equipped with a HP 7683 Injector and a HP5973 Mass Selective Detector. The injector temperature was held at 250 C. The initial oven temperature was 40 C for 3 min, the

temperature was then ramped to 280 C at 12 C/min and held at 280 C for 5 min. Helium was used as the carrier at a flow rate of 23.6 ml/min. A 30-m-long by 0.32-mm-i.d. DB-5 capillary column, which has a 0.25 μ m film thickness (J & W Scientific, Folsom, CA), was used. Selected ion monitoring (SIM) mode was used, which selected multiple characteristic target ions m/z 72.1, 91.1, 100.1, and 223.1 for DTB, and m/z 72.1, 100.1, 125, and 257 for TB.

2.2.8 Statistical analysis. Standard deviation, standard errors, and correlation coefficients were calculated using Excel 2000 (Microsoft Inc.). Dose-response data were analyzed using PROC NLIN (fitting log-logistic curve) procedure in SAS (SAS Institute Inc., SAS Campus Drive, Cary, NC) as described by Seefeldt et al. (1995).

2.3 Results

2.3.1 *In vitro* Test

2.3.1.1 Magenta Box *in vitro* test. Plant heights were charted against concentrations of TB or DTB on a log scale for TB (Figure 2-1A) and DTB (Figure 2-1B). TB and DTB appeared to stimulate Bengal rice growth at concentrations of 0.001-1 μ g/ml for TB, and at 0.001-0.1 μ g/ml of DTB. Plant heights were reduced at concentrations above 10 μ g/ml for TB and 1 μ g/ml for DTB. There was little difference in plant height for TB at concentrations of 10 and 100 μ g/g, and for DTB at concentrations of 1, 10, and 100 μ g/g. For Bengal rice grown in MS for 24 days, the values for ED50 were estimated as 0.4 μ g/ml for DTB and 3.8 μ g/ml for TB using log-logistic model analysis (Seefeldt et al. 1995).

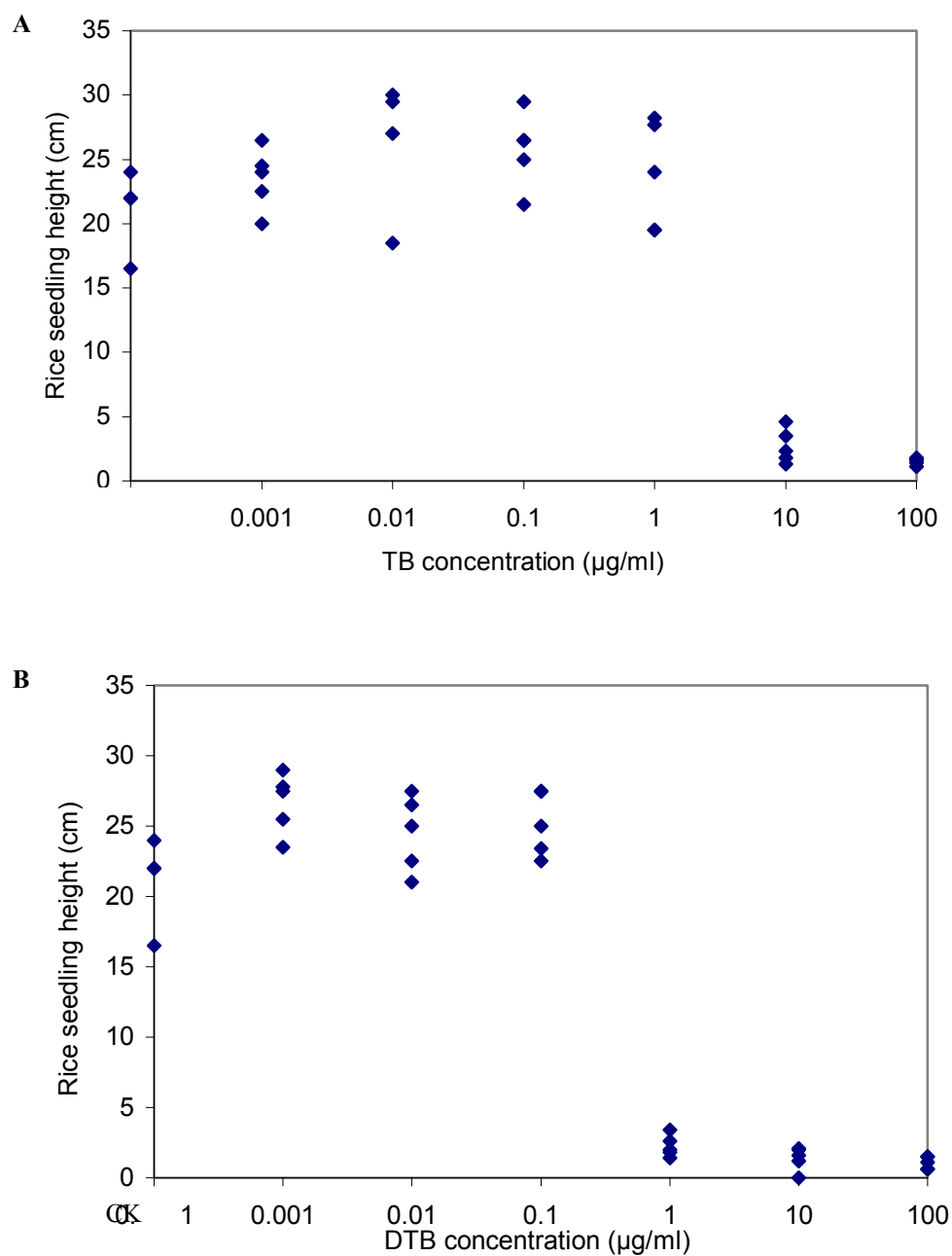


Figure 2-1. Dose-responses for thiobencarb (TB) and dechlorinated thiobencarb (DTB) on Bengal rice grown in Murashige-Skoog agar in Magenta boxes for 24 days. A. Scatter plot of rice plant heights on different concentrations of TB. B. Scatter plot of rice plant heights on different concentrations of DTB. Each treatment had five replicates.

2.3.1.2 Phytotoxicity of TB, DTB and their mixtures on rice. Dose-response curves of Lafitte rice to TB, at 12 days after treatment (DAT), were grouped by six levels of DTB exposure (Figure 2-2). Higher levels of TB reduced seedling height while the level of DTB remained constant, suggesting that the phytotoxic effects of TB and DTB to rice seedlings are additive. In the absence of DTB, the 0 TB treatment had the highest seedling height, approximately 9.4 cm. Exposure to 1, 2, and 5 µg/ml of TB significantly reduced rice seedling heights to 6.8, 6.8, and 6.1 cm, respectively. However, there was not a significant difference in rice seedling heights among these three treatments. Rice seedling height was significantly further reduced at 10 and 20 µg/ml of TB, to 3.6 and 1.6 cm, respectively. ED50 was estimated at 6.2 µg/ml for TB with 95% confidence interval (CI) from 3.3 to 9.1, and 0.3 µg/ml for DTB (95% CI: 0.2 – 0.5) using a log-logistic model. In the presence of low concentrations of DTB (0.1 and 0.2 µg/ml), rice seedling heights were more reduced than those in the absence of DTB at 1-10 µg/ml of TB. At these DTB concentrations, seedling heights were generally reduced as the concentrations of TB increased from 1-10 µg/ml of TB. The reductions at 10 to 20 µg/ml of TB were not significant at 20 µg/ml of TB, where the reductions in seedling heights were close to maximum. In the presence of higher concentrations of DTB (0.5, 1, and 4 µg/ml), rice seedling heights were generally lower than 2 cm. Reductions in seedling heights were not apparent as concentrations of TB increased from 0 to 20 µg/ml. One exception was at 0 µg/ml of TB where rice seedling height was less reduced at 0.5 µg/ml of DTB than by 1 or 4 µg/ml of DTB, but with any concentration of TB, rice seedling heights were highly reduced and were not separated from each other.

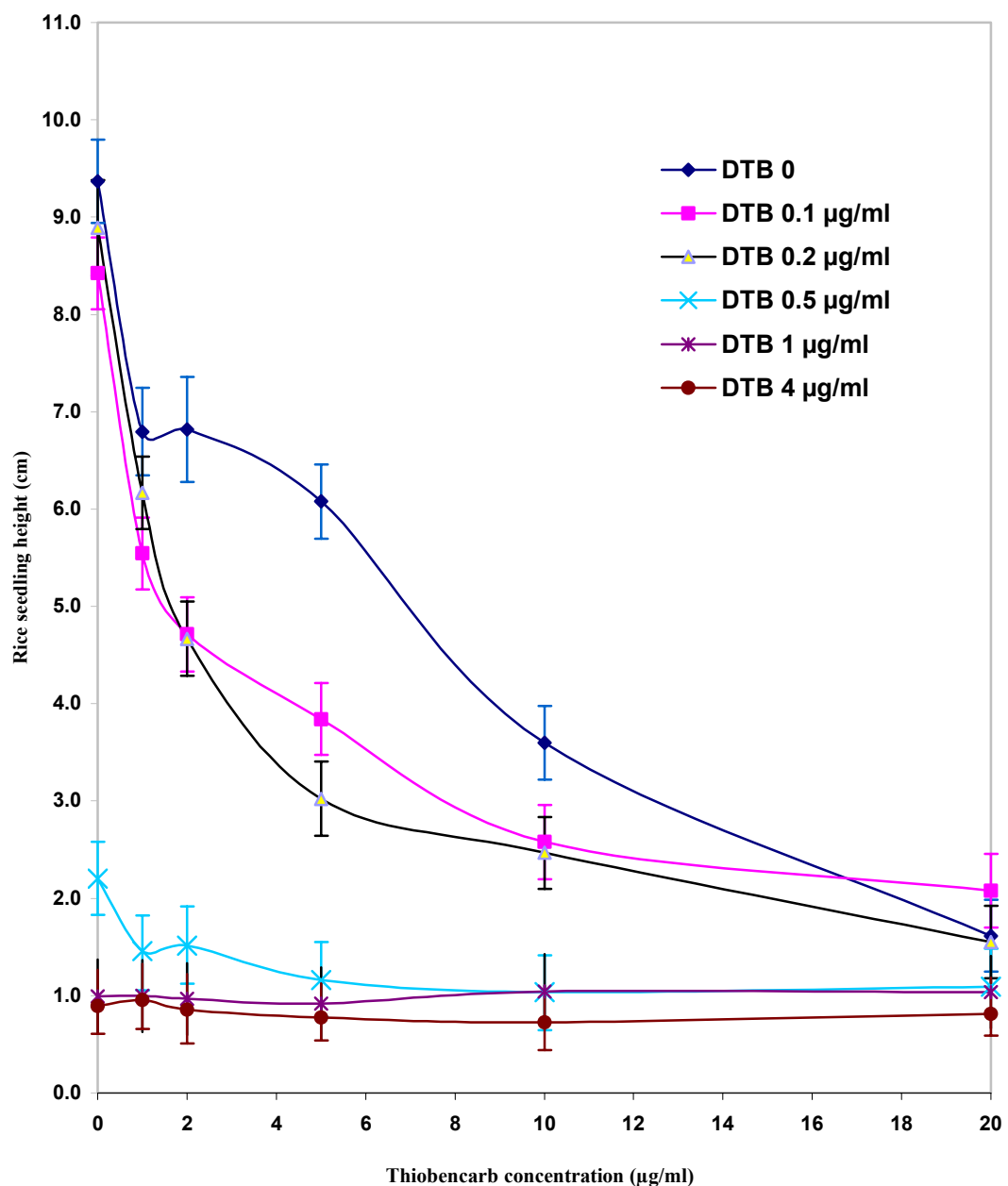


Figure 2-2. Dose-response curves for thiobencarb (TB) on Lafitte rice under the influence of different concentrations of dechlorinated thiobencarb (DTB). TB and DTB were incorporated into Murashige-Skoog agar medium at various concentrations. The height of rice seedlings was measured 12 days after transplanting. Error bars represent standard error with $n=40$.

The additive phytotoxicity of TB and DTB, when applied at less than 0.5 µg/ml of DTB or less than 20 µg/ml of TB, was also apparent when the treatments were grouped by the six TB concentrations (Figure 2-3). In the absence of TB, or in the presence of 1 µg/ml of TB, there was not a significant reduction of rice seedling heights for DTB at concentrations of 0, 0.1 and 0.2 µg/ml. However, the reduction was more significant as DTB increased to 0.5 µg/ml. Rice seedling heights remained lowest (approximately 1 cm) when DTB concentrations in the medium reached 1 µg/ml or higher, regardless of the concentrations of TB.

2.3.2 Tolerance of rice cultivars to DTB and TB. Generally, DTB had little inhibitory effect on rice seedlings when applied at 0.2 µg/ml compared to higher concentrations (Figure 2-4). Treated rice seedlings were indistinguishable from those of the control, with normal color and no leaf twisting. However, the phytotoxic effect was significant when the DTB concentration reached 1 µg/ml. Rice seedlings were dark green and shorter than the control. Some leaves were twisted. TB showed little inhibitory effect at 8 µg/ml, but its phytotoxic effects were significant at 40 µg/ml (Figure 2-5). After exposure to 40 µg/ml of TB, rice leaves also showed dark green color and twisting.

The inhibitory effects of TB and DTB on four different rice cultivars were compared in Table 2-2. In experiment 1, M201 was significantly less inhibited than Bengal, Cocodrie, and Lafitte at 0.2 µg/ml of DTB at 5 DAT. At 1 µg/ml of DTB, M201 was less inhibited than Bengal and Lafitte, but not significantly different from Cocodrie. At 8 and 40 µg/ml of TB, the inhibitory effect on M201 was significantly different from those on Cocodrie and Lafitte, but not from that of Bengal. However, this difference

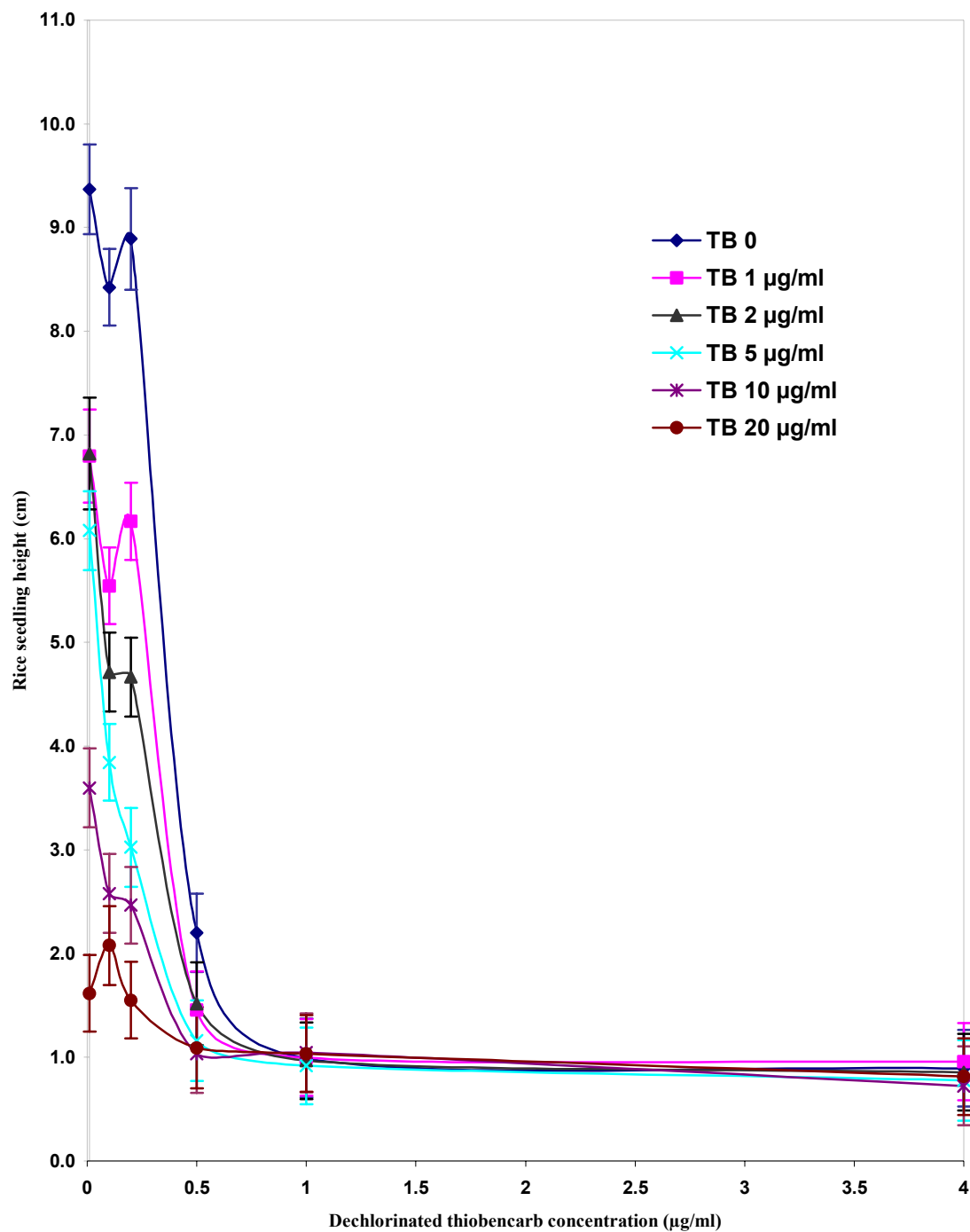


Figure 2-3. Dose-response curves for dechlorinated thiobencarb (DTB) on Lafitte rice under the influence of different concentrations of thiobencarb (TB). TB and DTB were incorporated into Murashige-Skoog agar medium at various concentrations. The height of rice seedlings was checked 12 days after transplanting. Error bars represent standard error with n=40.

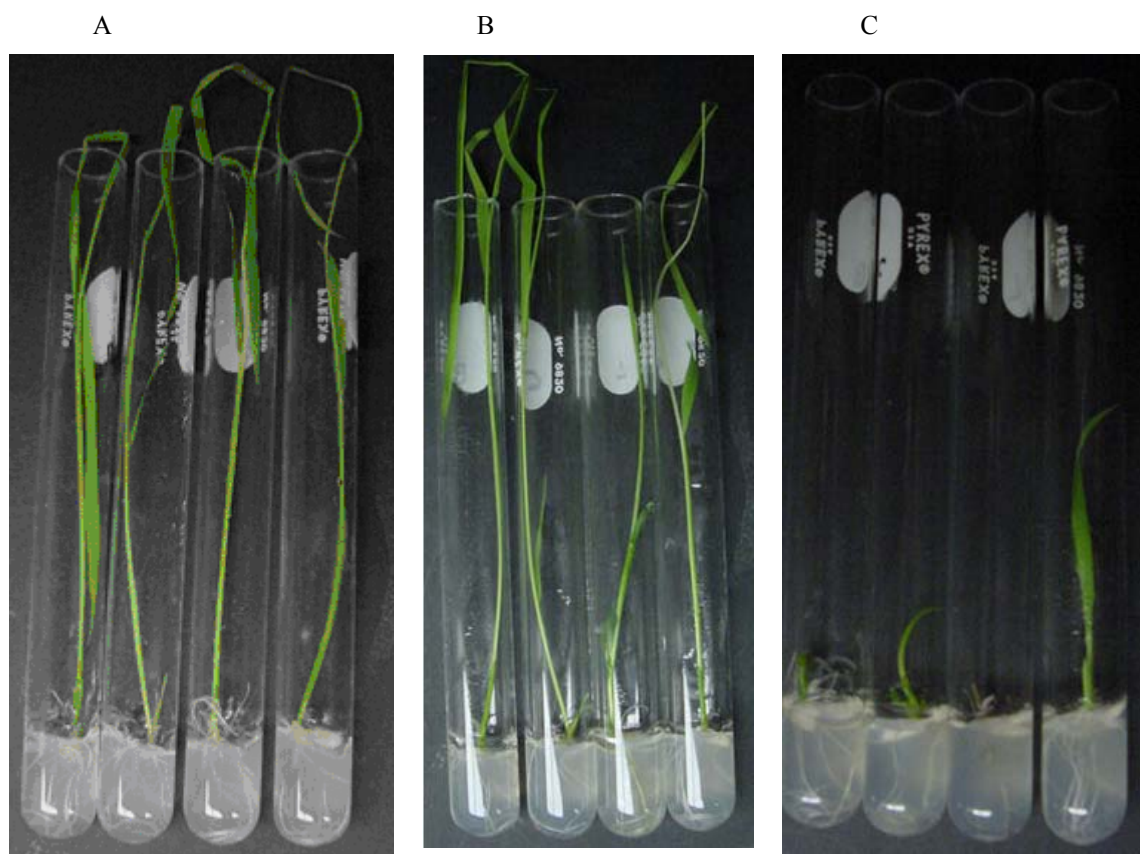


Figure 2-4. Seedlings of four rice cultivars growing in Murashige-Skoog agar medium with 0 (A), 0.2 µg/ml (B) and 1 µg/ml (C) dechlorinated thiobencarb incorporated into the medium (12 days after transferring). Test tubes from left to right in each photograph correspond to the rice cultivars Bengal, Cocodrie, Lafitte, and M201.

A



B



Figure 2-5. Seedlings of four rice cultivars growing in Murashige-Skoog agar medium with 8 $\mu\text{g/ml}$ (A) or 40 $\mu\text{g/ml}$ (B) thiobencarb incorporated into the medium (12 day after transferring). Test tubes from left to right in each photograph correspond to the rice cultivars Bengal, Cocodrie, Lafitte, and M201.

Table 2-2. Growth inhibition caused by thiobencarb (TB) and dechlorinated thiobencarb (DTB) in four rice cultivars growing in Murashige-Skoog medium

Experiment	Days after transfer (DAT)	Cultivar	Sample size (n)	DTB ^w		TB ^w	
				0.2 µg/ml	1 µg/ml	8 µg/ml	40 µg/ml
1	5	Bengal	8	19.0 a	93.1 a	47.1 b	87.6 b
		Cocodrie	8	22.8 a	85.3 ab	81.5 a	92.8 a
		Lafitte	8	32.7 a	94.9 a	69.3 a	93.0 a
		M201	16	4.8 b	79.6 b	36.3 b	87.4 b
	12	Bengal	8	20.5 a	92.1 a	24.1 c	90.9 a
		Cocodrie	8	7.7 a	91.7 a	83.4 a	94.0 a
		Lafitte	8	12.6 a	98.3 a	51.4 b	94.8 a
		M201	16	4.8 a	82.8 a	47.6 bc	86.1 b
2	6	Cocodrie	23	21.1 a	79.6 b	66.7 a	78.1 b
		Lafitte	16	19.0 a	87.8 a	76.7 a	84.0 a
		M201	24	6.5 a	84.9 a	71.4 a	84.0 a
	17	Cocodrie	23	15.0 a	95.0 a	80.7 a	91.9 a
		Lafitte	16	14.7 a	94.6 a	80.3 a	89.0 a
		M201	24	3.4 b	91.5 a	77.7 a	89.5 a

^w Data are percent inhibition of growth compared with seedling heights of the control.

^x Means followed by the same letters within columns in the same experiment/DAT and within the same concentration of DTB or TB are not significantly different (LSD, $p=0.05$). Statistical analyses were conducted following arcsine transformation of percent inhibition.

among the four cultivars was not significant at 12 DAT for 0.2 and 1 $\mu\text{g/ml}$ of DTB. At 8 and 40 $\mu\text{g/ml}$ of TB, M201 was less inhibited than Cocodrie.

In experiment 2, numbers of plants varied from 16 to 24 in each treatment. Among the three cultivars tested, M201 was less inhibited than the other three cultivars at 0.2 $\mu\text{g/ml}$ of DTB. The difference was most significant after 17 days. M201 did not show less growth inhibition than the other cultivars at 1 $\mu\text{g/ml}$ of DTB and at 8 and 40 $\mu\text{g/ml}$ of TB.

2.3.3 Greenhouse bioassay. Rice seedlings grew normally when exposed to low concentrations of DTB and TB in the greenhouse. Seedlings turned dark green and were stunted when high concentrations of TB and DTB were applied. Rice seedlings exposed to high DTB concentrations, such as 100 $\mu\text{g/g}$ (dry soil basis), had only 1-2 thin leaves remaining (Figure 2-6).

Dose-response curves for DTB effect on seedling height were similar for autoclaved soil and the control (nonautoclaved soil) in the range of 0.01 to 0.1 $\mu\text{g/g}$ of DTB (Figure 2-7A). At 1 $\mu\text{g/g}$ of DTB, the rice seedling heights in autoclaved soil were inhibited less than those in nonautoclaved soil. The inhibitions became similar at 10 $\mu\text{g/g}$ of DTB. At 100 $\mu\text{g/g}$ of DTB, the rice seedling heights in autoclaved soil were inhibited more than those in the nonautoclaved soil. The inhibition of rice seedling heights was 52% for autoclaved soil and was 40% for nonautoclaved soil.

The dose-response curves for DTB effect on total fresh weight were similar for autoclaved soil and nonautoclaved soil at the range of 0.01-1 $\mu\text{g/g}$ DTB, as indicated by the overlapping of the error bars (Figure 2-7B). At 10-100 $\mu\text{g/g}$ of DTB, rice fresh weight

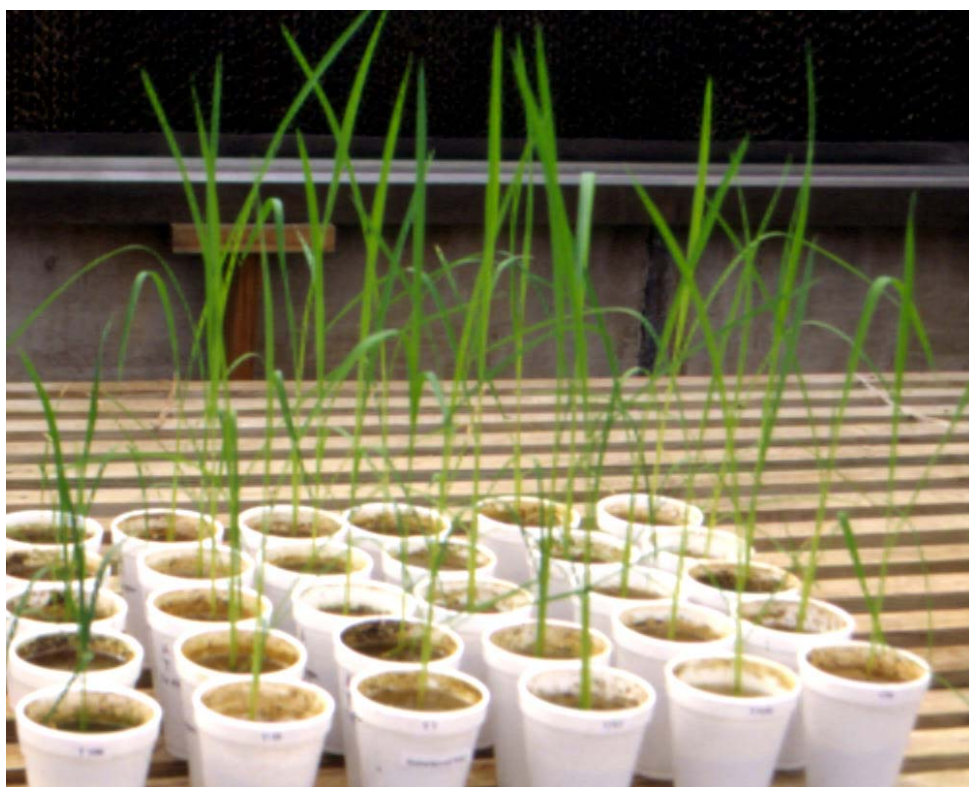


Figure 2-6. Seedlings of Bengal rice growing in autoclaved soil amended with various concentrations of thiobencarb (TB) or dechlorinated thiobencarb (DTB) in a greenhouse study. Top: DTB concentrations from left to right 100, 10, 1, 0.1, 0.01, and 0 $\mu\text{g/g}$ (dry soil basis), Bottom: TB concentrations from left to right 100, 10, 1, 0.1, 0.01, and 0 $\mu\text{g/g}$ (dry soil basis).

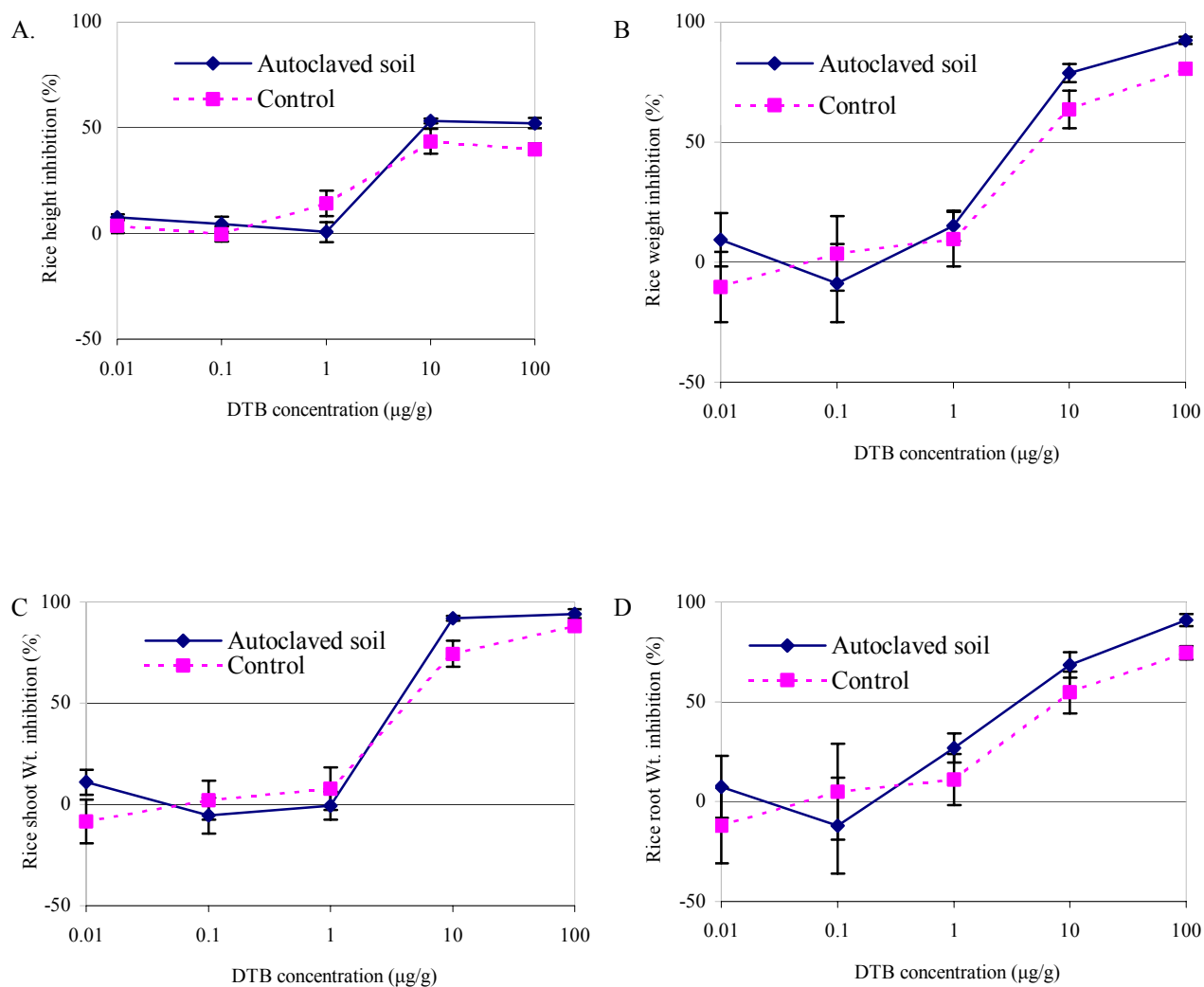


Figure 2-7. Effect of dechlorinated thiobencarb (DTB) on plant height (A), total fresh weight (B), shoot fresh weight (C) and root fresh weight (D) of seedlings of Bengal rice grown in autoclaved soil and nonautoclaved soil (control) in greenhouse bioassays. Results are the average values of five replications. Bars represent standard errors.

was less in autoclaved soil than in nonautoclaved soil. The highest inhibition occurred at 100 µg/g of DTB for both autoclaved soil (92%) and nonautoclaved soil (81%).

Reduction in shoot fresh weight for autoclaved soil and nonautoclaved soil across the range of DTB concentrations were similar to the dose-response curves based on total fresh weight (Figure 2-7C). The inhibition rates were not different at 0.1-1 µg/g DTB for both types of soil. Rice fresh shoot weights were inhibited more in autoclaved soil than in nonautoclaved soil when DTB increased to 10 µg/g or higher. The highest inhibition rate was 94% for autoclaved soil and was 88% for nonautoclaved soil at 100 µg/g DTB.

The dose-response curves for DTB effect on root fresh weight were similar for autoclaved soil and nonautoclaved soil in the range of 0.01-10 µg/g of DTB (Figure 2-7D). At 100 µg/g of DTB, root fresh weights were inhibited more in the autoclaved soil (91%) than those in the nonautoclaved soil (74%).

Rice seedlings were generally inhibited less by TB than by DTB in the greenhouse bioassays. The dose-response curves for TB's effect on rice heights were similar for autoclaved soil and nonautoclaved soil in the range of 0.01-1 µg/g of TB (Figure 2-8A). Rice seedling heights were inhibited more at 10 µg/g of TB in nonautoclaved soil. This difference was not apparent at 100 µg/g of TB. The highest inhibition was 45% for autoclaved soil and 40% for nonautoclaved soil.

The dose-response curves for TB effect on total fresh weight were similar for autoclaved soil and nonautoclaved soil as indicated by the overlapping of the error bars across the range of TB concentrations. The highest total fresh weight inhibition was 64% for autoclaved soil and 54% for nonautoclaved soil.

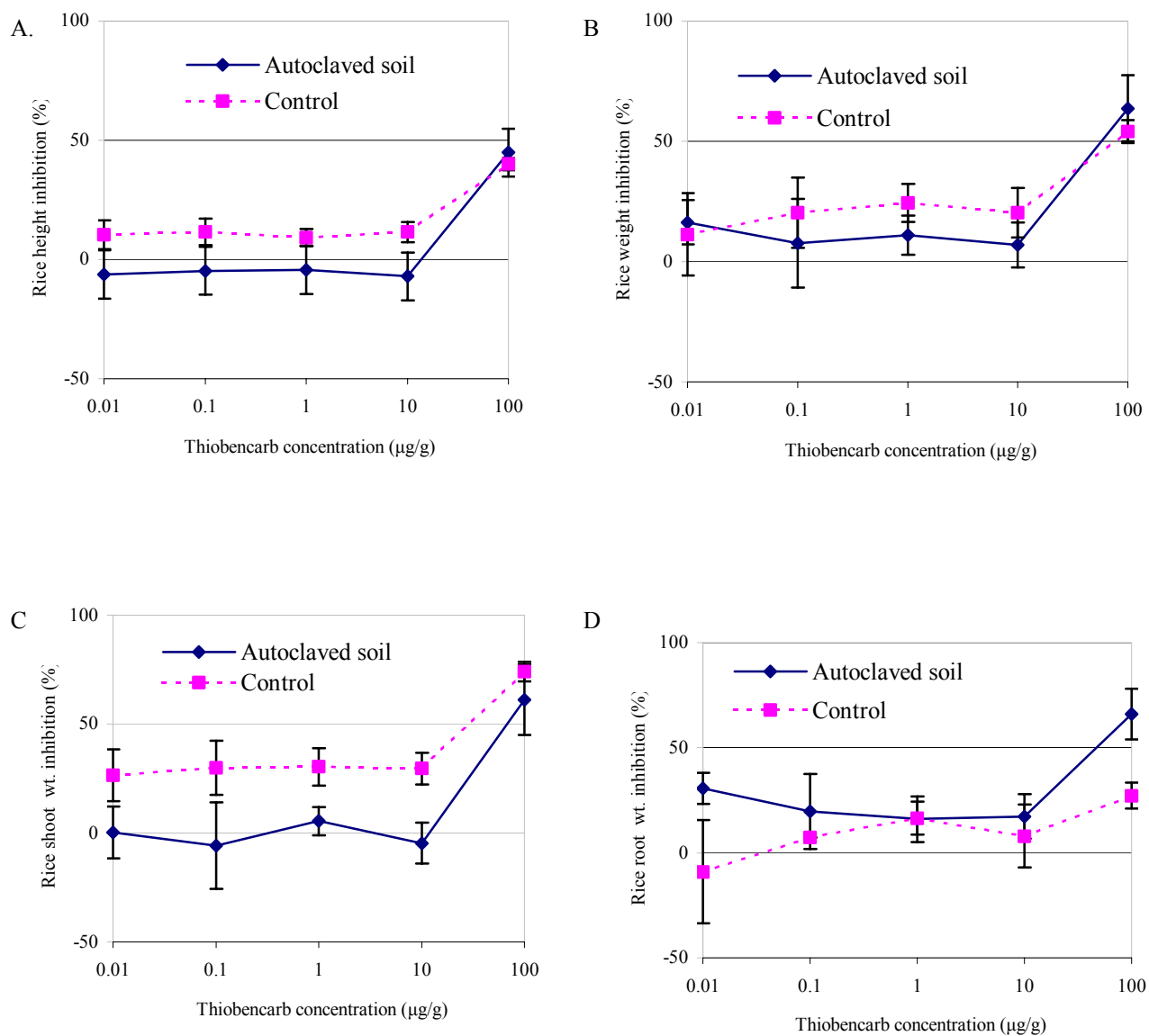


Figure 2-8. Effect of thiobencarb on plant height (A), total fresh weight (B), shoot fresh weight (C) and root fresh weight (D) of seedlings of Bengal rice grown in autoclaved soil and nonautoclaved soil (Control) in greenhouse bioassays. Results are the average values of five replications. Bars represent standard errors.

The dose-response curves for TB effect on shoot fresh weights were different for autoclaved soil and nonautoclaved soil (Figure 2-8C). The total fresh weight inhibition rates were significantly higher for nonautoclaved soil than autoclaved soil for 0.01 to 10 $\mu\text{g/g}$ of TB. This difference became less apparent at 100 $\mu\text{g/g}$ of TB. The highest inhibition rate was 74% for nonautoclaved soil and 61% for autoclaved soil.

The dose-response curves for TB effect on root fresh weight were different for autoclaved soil and nonautoclaved soil at either 0.01 $\mu\text{g/g}$ or 100 $\mu\text{g/g}$ (Figure 2-8D). Rice root fresh weights were inhibited more in autoclaved soil than in nonautoclaved soil. There were no differences for autoclaved soil and nonautoclaved soil in the range of 1-10 $\mu\text{g/g}$ of TB. The highest root fresh weight inhibition rate was 66% in autoclaved soil and 27% in nonautoclaved soil.

Data from soil bioassays were fitted to log-logistic model to estimate ED50. In autoclaved soil, the values of ED50 for DTB were estimated at 3.1 (95% CI: 0 – 6.1), 3.1 (95% CI: -1.8 – 8.0), and 2.9 (95% CI: - 1.8 – 7.6) $\mu\text{g/g}$ for total fresh weight, and shoot fresh weight, and root fresh weight, respectively. In nonautoclaved soil, values of ED50 for DTB were estimated at 3.8 (95% CI: -1.7 – 9.2), 3.4 (95% CI: 0.1 – 6.7), and 1.1 (95% CI: 0.7 – 1.4) $\mu\text{g/ml}$ based on total fresh weight, shoot fresh weight, and root fresh weight, respectively. Data from the plant heights in some DTB treatments and all TB treatments were not convertible by the log-logistic model and no ED50 was estimated.

2.3.4 Quantification of TB and DTB concentrations in plants and soils and phytotoxicity to rice plants in a conducive soil. When the SIM mode was chosen, GC/MS was very sensitive for detecting TB and DTB, even at low concentrations in soil suspensions (Figure 2-9). Under the conditions used, TB had a retention time of

13.01 min while DTB had a retention time of 12.01 min. The baseline was very low, and the peaks of TB and DTB were very clear. The detection limit was 0.01 µg/ml for both TB and DTB. The same procedure was used for all quantifications of TB and DTB in plant tissues, soil suspensions, and soil extracts.

When TB was applied to conducive field soil, DTB was produced quickly and rice plants usually died. Remaining plants were stunted and dark green (Figure 2-10). In the greenhouse study using this conducive soil, rice plants showed DPS 2-3 weeks after transplanting, and the number of DPS plants increased with time. Figure 2-11 shows healthy plants and DPS plants after they were removed from the pots and washed with tap water at 41 DAT. Some treated plants had more tillers than the healthy plants while others had fewer tillers. Roots of the DPS plants were indistinguishable from those of the healthy plants. Samples of soil, leaf/culms, and roots were extracted for GC/MS quantification of TB and DTB, and the results are shown in the table in Figure 2.11. In the control, TB and DTB were not detected in the soil or leaf/culm samples. Only trace amounts (0.02 µg/g) of TB was detected in the root sample, probably as a result of contamination. In the three treatments with TB applied, almost all TB was transformed into DTB, with only a trace amount of TB remaining in the soil (0.01 µg/g) and leaf (0 to 0.03 µg/g). However, there was more DTB in the soil (from 0.31 to 0.55 µg/g) than in leaves (0.02 to 0.06 µg/g) or in the roots (0.10-0.24 µg/g).

2.3.5 Phytotoxicity of DTB transformed from TB in conducive soil.

When rice plants were grown in the greenhouse in DPS-conductive soil treated with TB, the concentrations of TB and DTB in the soils were quantified and correlated to

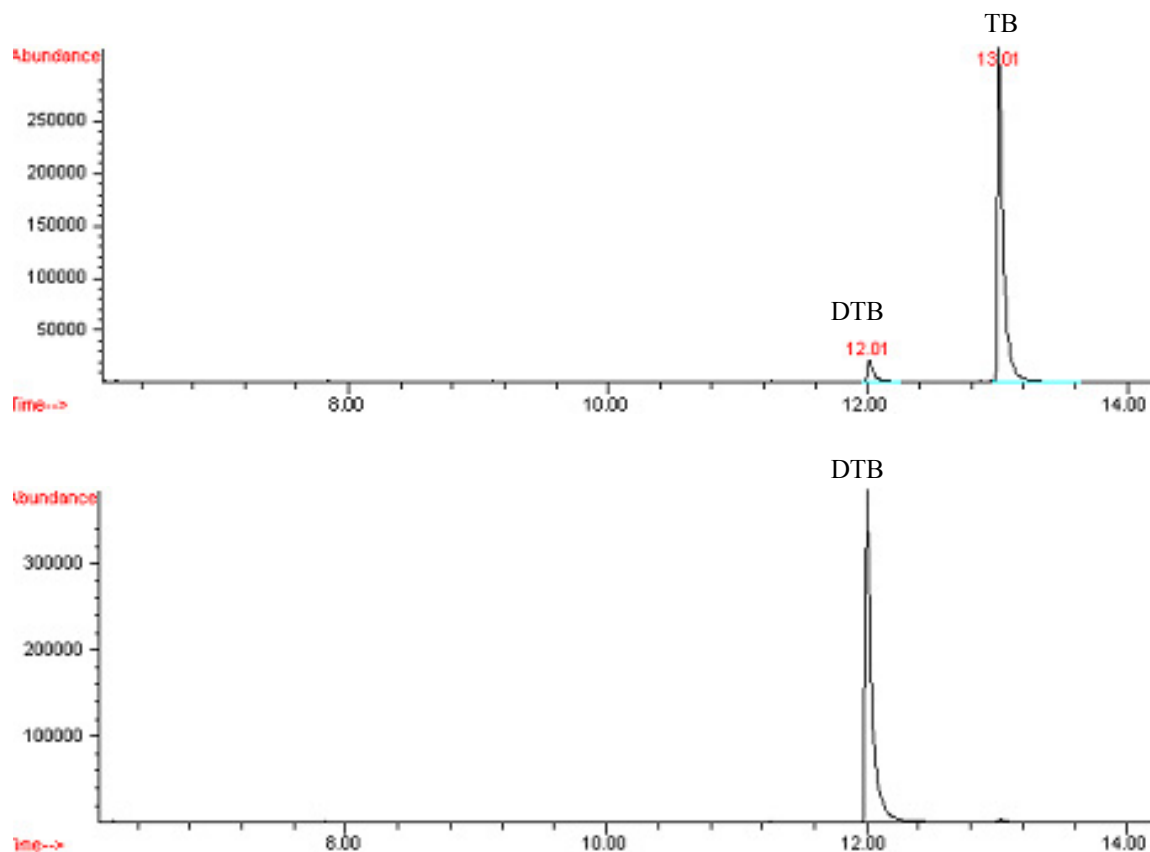


Figure 2-9. Chromatograms showing TB and DTB concentrations at different sampling times in a soil suspension. Top. At the beginning, TB concentration was high (10 $\mu\text{g/ml}$ TB was added) while DTB concentration was low. Bottom. After incubation, TB disappeared and DTB increased.

plant height, total fresh weight, and tiller numbers. The phytotoxicity caused by TB and DTB was compared by scatter plots of plant heights, tiller number, or total fresh weight against the concentrations of DTB (Figure 2-12) and the concentrations of TB (Figure 2-13). Plant height showed a significant negative correlation ($r = -0.72$, $P < 0.0001$) with increasing concentrations of DTB. Total fresh weight also showed significant negative correlation with the concentration of DTB, with a correlation coefficient $r = -0.48$ ($P = 0.009$). Tiller number showed a marginally significant negative correlation with $r = -0.37$ ($P = 0.055$).



Figure 2-10. Phytotoxicity to Bengal rice caused by dechlorinated thiobencarb (DTB) produced from thiobencarb in field plots in the South Farm Unit, Rice Research Station, Crowley, LA. Top: Non-treated control. Bottom: Treated with thiobencarb (Bolero 10 G at the rate of 4.5 kg ai/ha).



Plant	Treatment	Soil		Leaf/culms		Root	
		DTB	TB	DTB	TB	DTB	TB
1	Control	0	0	0	0	0	0.02
2	TB-treated	0.31	0.01	0.02	0	0.14	0.08
3	TB-treated	0.33	0.01	0.03	0.03	0.10	0.03
4	TB-treated	0.55	0.01	0.06	0	0.24	0.03

Figure 2-11. Phytotoxicity effects of thiobencarb (TB), applied as Bolero 8 EC at 4.5 kg ai/ha rate, and dechlorinated thiobencarb (DTB), produced in the soil from TB, on Bengal rice in greenhouse bioassay in a conducive soil. The table on the bottom shows TB and DTB concentrations ($\mu\text{g/g}$) detected from the soil where the plant was growing, leaf/culm, and roots at 41 days after TB application. The plant numbers in the table correspond to the number in the graph.

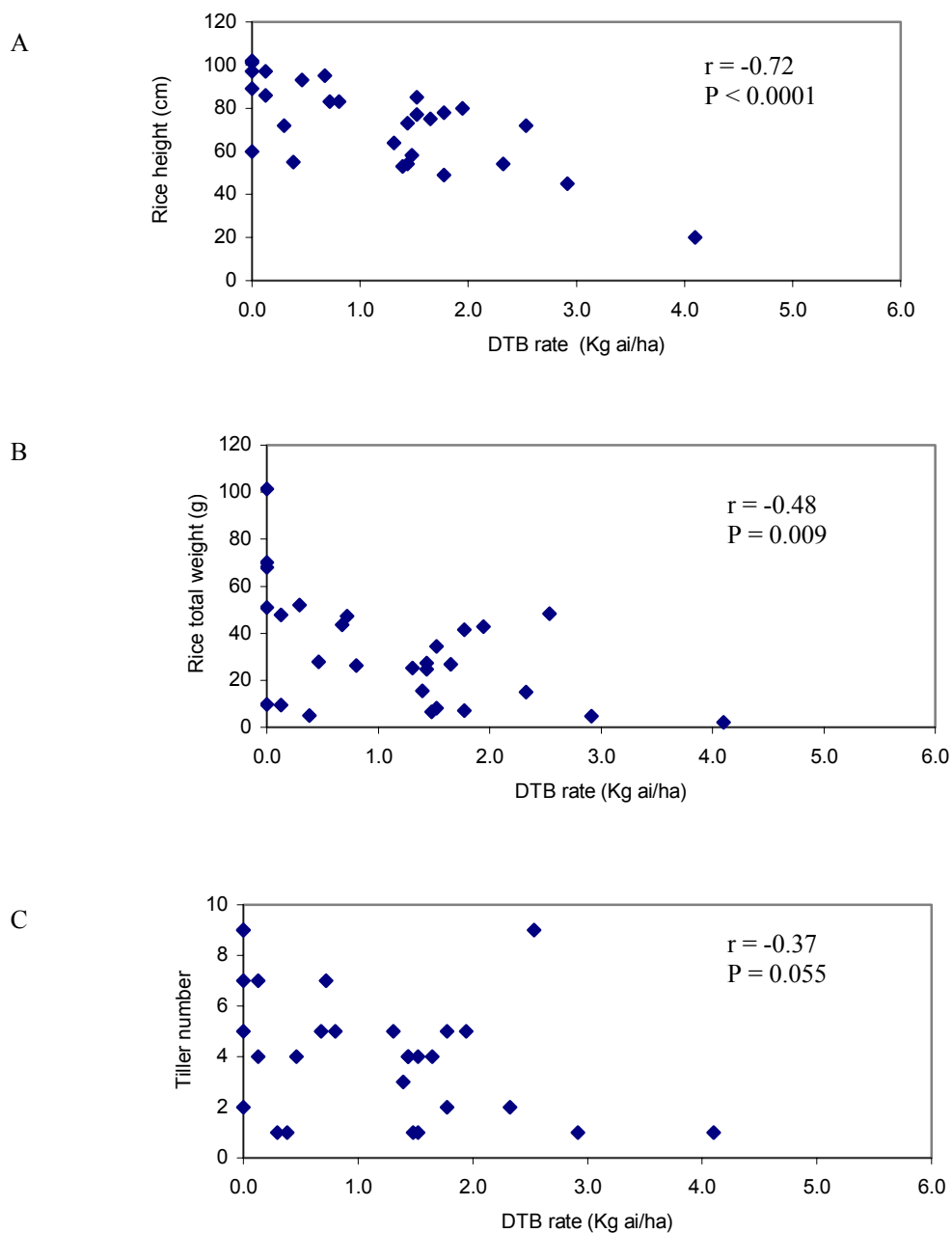


Figure 2-12. Scatter plot for effect of dechlorinated thiobencarb (DTB) concentrations in soil on rice plant height (A), total fresh weight (B) and tiller number (C) in greenhouse study using conducive soil. Data were collected 41 days after treatment.

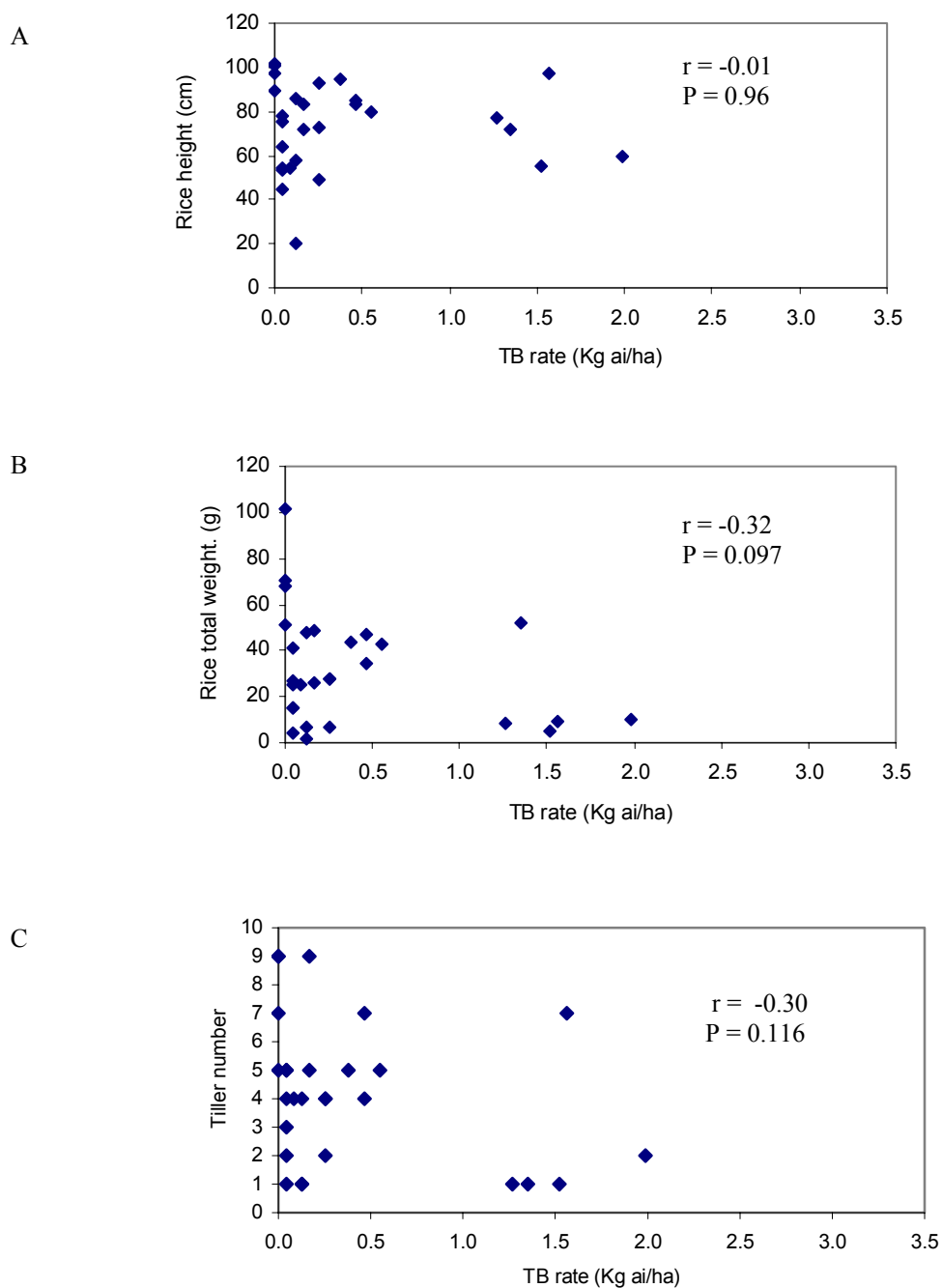


Figure 2-13. Scatter plot for effect of thiobencarb (TB) concentrations in soil on rice plant height (A), total fresh weight (B) and tiller number (C) in greenhouse study using conducive soil. Data were collected 41 days after treatment.

When the plant height was plotted against TB concentration, height was not affected by increasing TB concentration ($r = -0.01$, $P = 0.96$). The correlations were not significant between tiller number and TB concentration ($r = -0.30$, $P = 0.116$) and between total fresh weight and TB concentration ($r = -0.32$, $P = 0.097$).

2.3.6 The persistence and of TB and DTB in rice plant. When rice seedlings were exposed to equal concentrations of TB and DTB, they absorbed and accumulated TB and DTB at different rates (Table 2-3). Rice appeared to absorb more TB than DTB. The plants accumulated more TB than DTB in their roots up to 4 days after exposure to the chemicals. TB concentrations also decreased more quickly than DTB in the aqueous solution in which the rice seedlings were incubated for 1 day.

Table 2-3. Concentrations ($\mu\text{g/g}$) of thiobencarb (TB) and dechlorinated thiobencarb (DTB) in rice seedlings and soaking solution four days after exposure to TB and DTB aqueous solutions^W

Source of extraction	TB	DTB	P-value ^Y
Rice leaf	0.01 (0.01)	0	0.856
Rice root	0.29 (0.03)	0.21 (0.04)	0.006
Differences in TB and DTB in aqueous solution before and after the soaking ^X	1.10 (0.03)	0.95 (0.02)	<0.0001

^W Rice seedlings (cv. Bengal, 2-3 leaf stage) were dipped into water containing 1 $\mu\text{g/ml}$ of both TB and DTB for 24 h and then washed with tap water and incubated in fresh tap water for 4 d before subjected for extraction. Numbers in the parenthesis are standard deviations.

^X Water samples were collected before and after the dipping of rice seedlings and extract for TB and DTB to obtain the different concentrations.

^Y Statistical comparisons were performed using SAS Proc GLM procedure and LSMeans. P-value in each row is the P-value for testing difference between TB and DTB.

2.4 Discussion

In this study, the phytotoxicity of TB, DTB and mixtures of the two were evaluated through *in vitro* and greenhouse bioassays. The *in vitro* study using Magenta boxes to

test the phytotoxicity of TB and DTB on Bengal rice estimated ED₅₀ values between 1 to 10 µg/ml for TB and 0.1 to 1 µg/ml for DTB. At low concentrations, both TB and DTB appeared to have slight stimulative effects on the growth of rice seedlings. This phenomenon is also true for many other herbicides and was coined the “Hormetic effect” (Schabenberger et al. 1999). DTB was more toxic to rice than TB, and the damage to rice by DTB had more effect on the height and total fresh weight than on total root weight. TB and DTB appeared to be additive in inhibiting rice seedling growth at lower rates of TB. A synergistic effect between TB and DTB was not observed in these experiments. Where TB and DTB are at different concentrations in the soil and in plants during the process of dechlorination, DTB is more likely the cause of DPS in the fields based on our toxicity data.

Among the four rice cultivars selected for the *in vitro* study, there were differences in the phytotoxicity responses to TB and DTB. M201 was significantly more tolerant than the other three cultivars to DTB at low concentrations. The inhibitory effect on all cultivars was most significant 17 DAT. The tolerance of M201 was less when DTB concentrations increased to 1 µg/ml. The tolerance of M201 suggests that further studies should be conducted to determine if it is possible to select rice cultivars that are resistant or tolerant to DTB. It is interesting that M201 was developed by the breeding program in Biggs, California where thiobencarb herbicide has been used for many years.

Soil bioassays are important because they are closer to field conditions. However, soil bioassays sometimes can be difficult to interpret because results can be complicated by many factors such as soil characteristics, microbial, and nutritional conditions. TB and DTB were absorbed by organic matter and clay components of the soil (Braverman et al.

1990; Mabury et al. 1996). Furthermore, photodegradation (Chen et al. 1982) and microbial degradation (Ishikawa et al. 1976; Ishikawa et al. 1977) may have affected availability of TB and DTB in the greenhouse study. Because TB and DTB are more persistent under anaerobic conditions (Nakamura et al. 1974; Tatsuyama et al. 1981), soil used in the soil bioassays were flooded for 14 days before TB or DTB were applied. The flooding of soil facilitates the formation of anaerobic zones. Immediately after the application of TB or DTB, the soil and water were mixed to create a suspension, thus helping TB and DTB to be adsorbed onto soil components. These steps helped to reduce the loss of TB and DTB and ensured that seedlings were exposed to the chemical.

Greenhouse bioassays using autoclaved and nonautoclaved nonconductive soil produced different dose-response curves for TB. TB appeared to be more toxic in nonautoclaved soil, especially when shoot fresh weights were compared. This suggested that, although the soil appeared to be nonconductive in field tests, that is, no obvious symptoms were produced, it is probable that some DTB was produced in this treatment and recorded as toxicity by TB. The toxicity of DTB to rice seedlings was significantly reduced for nonautoclaved soil at 10-100 $\mu\text{g/g}$ of DTB compared to autoclaved soil. This reduced phytotoxicity may have resulted from the degradation of DTB by native soil microorganisms. This is possible as DTB was degraded quicker than TB in soil suspensions under aerobic conditions (Chapter 4). Also, the autoclaving treatment of soil may have altered the structure of soil particles, and this might have an impact on the adsorption and desorption of TB or DTB to soil particles (Braverman et al. 1990). The autoclaving treatment killed most native soil organisms, but microorganisms were soon re-established after the soil was exposed to the air and tap water. On the other hand,

autoclaving may have released some nutrients from the soil particles as the plants were larger than the nonautoclaved treatments. This was corrected for by plotting percent inhibition instead of direct measurements.

The experiments in the conducive soil differed from those in the non-conductive soil in several aspects. First, only TB was directly applied into each pot. The DTB that the rice plants were exposed to was transformed from TB by reductive dechlorination, which usually takes 2 to 4 weeks to complete. Thus, rice plants were not exposed to DTB or only exposed to very low concentrations of DTB at the early stages. Second, rice plants were exposed to both TB and DTB when dechlorination was not been completed. The inhibition of rice seedling growth was more immediate, and results appeared to be more uniform. However, in the conducive soil bioassays, TB was applied at the time the rice seedlings were transferred into the pot. The rice seedlings were exposed to TB until reductive dechlorination of TB occurred. The dechlorination rates differed from pot to pot, even with the same treatment, presumably due to different microbial activities. Rice plants were exposed to various TB and DTB concentrations at different growth stages, resulting in high variation among the data. Nevertheless, this study showed that high concentrations of DTB contributed to shortened plant height and reduced total fresh weight. These observations were also supported by a correlation analysis, where DTB concentrations were found to be negatively correlated to rice plant height, while TB concentrations were not significantly correlated. Tillering was not significantly affected in these experiments, possibly because tillering took place before DTB had accumulated to toxic levels.

The development of a method to quantify TB and DTB using GC/MS detection enabled the quick and accurate quantification of TB and DTB in soil and plant samples. This procedure was more useful for soil samples than plant tissues. Some compounds from rice plants may also be extracted and may have interfered with detection. Root samples appeared to contain more TB and DTB than leaf/culm samples. This probably relates to the absorption, translocation and metabolic patterns of TB and DTB in the plants. TB has been shown to be readily absorbed by roots of rice plants and translocated throughout the plant (Nakamura et al. 1974). Nakamura et al. (1974) reported that from 2 DAT to 4 DAT, TB concentration changed from 107 to 39 ($\times 10^2$ dpm/g) in rice leaves and stems, from 192 to 122 in rice roots; from 47 to 25 in barnyardgrass leaves and stems, and from 25 to 267 in barnyardgrass roots. Since TB is rapidly metabolized by the plant, it is expected that little residual TB would be detected from leaves/culms.

The preliminary test with persistence of TB and DTB in rice plants suggested that rice seedlings absorb and translocate DTB at a slower rate than they absorb and translocate TB. This was confirmed by the observation that DTB dissipated slower than the TB from aqueous solutions and that rice roots accumulated greater amounts of TB than DTB. Since DTB is at least ten-fold more toxic to rice plants than TB, mechanisms other than absorption and translocation may have contributed to the increase in phytotoxicity. It is likely that DTB by itself is more toxic to rice than TB. Further studies with better controlled conditions are necessary to address this problem.

Chapter 3

Relationship between Reductive Dechlorination of Thiobencarb and Redox Potential in a Soil Column

3.1 Introduction

One of the most important cultural practices in rice production is to flood the field to control weeds and conserve nitrogen. Because of limited O₂ penetration, a flooded soil can be differentiated into a surface oxidized layer and an underlying reduced layer (Reddy et al. 1980). The major factors determining the thickness of the oxidized surface layer include the supply of O₂ at the soil surface and the consumption rate in the soil.

Redox potential, which is a measure of the electron availability or potential, can be very useful in characterizing the two layers existing at the surface of flooded soil (Patrick et al. 1996). A platinum (Pt) electrode readily transfers electrons either to or from the medium, but presumably does not react chemically with the medium. When a Pt electrode is coupled with a suitable half cell of known potential, reducing systems tend to transfer electrons to the electrode while oxidizing systems tend to take electrons from the electrode. The redox potential corrected with hydrogen (Eh) is a common expression of soil redox potential, as it is readily determined with a platinum electrode. Along with pH, redox potential (Eh) defines conditions under which important biogeochemical processes take place (Patrick et al. 1996).

A well-oxidized soil has an Eh around +400 to +700 mV, a flooded soil may exhibit Eh's as low as -250 mV or even lower (Wang et al. 1993). A virtually O₂-free rice soil (except at the interface) can be found at a redox of around +350 mV (Reddy et al. 1980). A flooded rice field creates anaerobic zones characterized by microbial activities including fermentation, sulfate reduction or methanogenesis. These processes require the

constant presence of electron acceptors to sustain activity. The electron acceptors that have been reduced in the bulk soil can be regenerated by chemolithotrophic bacteria in the oxygenated zones. Generally, O_2 is reduced before other oxidants in soil.

Flooding rice soil also affects the pH of the soil. The pH of both acid and alkaline soils tends to converge to neutral pH after flooding (Patrick et al. 1985). Crowley silt loam soil, on which much of the rice in Louisiana is produced, is acidic with an original soil pH of 5.7 (Patrick & Jugsujinda 1992). After submergence of the soil, its pH increases to 6.9. Under reduced conditions and a neutral pH, methanogenic bacteria appear to be very active (Masscheleyn et al. 1993). The maximum CH_4 production rate was observed at pH 6.9, Eh -230 mV (Patrick et al. 1985).

Patrick and Delaune (1972) developed a technique to measure the redox potential profile of a flooded soil. A small Pt electrode was constructed by sealing 18 gauge Pt wire into 5 mm diameter Pyrex tubing with 1 mm of the wire extending from the tip. This Pt electrode was driven downward through an undisturbed flooded soil at a rate of 2 mm/h. In addition to a Pt electrode, a saturated calomel electrode was used to complete the cell so that measurements could be made with a pH meter. By using this system, the redox potential profiles of a Crowley silt loam surface soil at various incubation times after flooding were obtained. One major problem with this apparatus was related to the slow equilibrium rate of Pt at low redox potential values in a biologically active medium. To obtain a reliable redox reading at a given depth, it took several hours for the Pt to equilibrate. The speed of 2 mm/h for the Pt electrode might have caused the reading to be higher than actual redox values in reduced conditions, e.g., the lowest redox potential value for a flooded Crowley silt loam was around 0 mV for incubation from 1 day

throughout 13 weeks (Patrick and Delaune 1972). Banker et al. (1995) developed a modified method to measure redox profiles of a soil core. A Pt electrode was held stationary and the core was raised in mm intervals. Upon initial contact, the Pt electrode and soil surface were allowed to stabilize for 24 h. Subsequent redox readings were taken at 2-h intervals. This method improved the measurement of redox potentials as indicated by the redox value near -150 mV in a soil core collected from a rice field located at the Rice Research Station in Crowley, Louisiana. However, the soil core in this system had to be moved vertically in order to measure the redox profiles. The vertical movement of the soil core may have caused mixing of the soil layers.

The reductive dechlorination of rice field herbicide thiobencarb (TB) was first reported in 1980 in Japan from rice fields that previously had TB applied (Ishikawa et al. 1980). Dechlorinated thiobencarb (DTB) was shown to be very phytotoxic to rice plants. This disorder was also reported in Louisiana rice (Groth & Sanders 1996) and in California (Tjeerdema & Crosby 2000). The phytotoxicity associated with the transformation of TB into DTB was termed delayed phytotoxicity syndrome (DPS) by Groth & Sanders (1996). In Japan, fields associated with DTB were not widespread since many rice soils were not capable of supporting the dechlorination of TB (Ishikawa et al. 1980; Moon & Kuwatsuka 1984). A preliminary study using several California rice soils incubated with TB under various conditions, including the use of flooding and the addition of organic matter, also failed to produce any DTB, even after extended incubation times (Cheah & Crosby 1995). Observations in Louisiana, however, suggested that the disorders associated with DPS were spreading. DPS-affected rice increased to 8,094 ha by 1993 (Groth et al. 1999).

As with reductive dehalogenation of other halogenated aromatic compounds, the reductive dechlorination of TB requires reduced (anaerobic) conditions (Mohn & Tiedje 1992; Moon & Kuwatsuka 1985b). Information regarding the quantitative relationship of reductive dechlorination of TB and redox conditions is not available. In a study using a soil suspension prepared from DPS-conducive rice soil in Japan, Moon and Kuwatsuka (1985 b) measured redox potential by placing a Pt electrode at the bottom of a test tube containing diluted soil suspension. The changes in concentrations of TB and DTB in the suspensions were determined by sampling periodically from the test tubes. The results indicated that reductive dechlorination of TB only occurred in test tubes in which redox potential (Eh) fell below -200 mV. A control with Eh values higher than -100 mV through the incubation period failed to produce DTB (Moon & Kuwastuka 1984). These results should be considered preliminary because the specific position where dechlorination of TB occurred was not shown and the conditions at this location were not determined.

Understanding the relationship between reductive dechlorination of TB and redox conditions in rice soil could be useful in the management of DPS. It would also be helpful in locating TB-dechlorinating organisms by pinpointing the optimized TB-dechlorinating zone in a soil column. The objectives of this study were: 1) to develop a model system to test dechlorination by different microorganisms under controlled conditions; 2) to construct an apparatus to accurately measure the redox potential in different depths of a soil column; and 3) to determine the relationship between redox potential and reductive dechlorination of TB and to identify the most likely area in a soil column to isolate dechlorinating organisms.

3.2 Materials and Methods

3.2.1 Chemical and Soil. Technical grade TB (96.7%) was provided by Valent's Dublin Laboratory (Dublin, CA). DTB (98.9%) was provided by Kumiai Chemical Industry C., Ltd., Tokyo, Japan.

Soil was collected from the top 10 cm of a rice field at the Louisiana State University Ag Center Rice Research Station, near Crowley, Louisiana, which had been identified as conducive for dechlorination of TB (Bollich et al. 1996). The soil type was a Crowley silt loam, with the following properties: pH 5.6, organic matter 1.18%, and extractable nutrients (mg/kg): P (67-75), Na (66-68), K (62-69), Ca (1037-1100), Mg (229-238), Zn (4.91-5.91), S (9.19-9.3), As (4.64-4.74), and Fe (183-185.7). Soil was collected during the early spring when the field was dry, then spread on a table inside a greenhouse to dry further. The dry soil was then ground into powder and stored at 5 C until used.

3.2.2. Determining TB and DTB concentrations in partitioned soil columns.

3.2.2.1 Effects of timing of application of TB on DTB formation in a syringe test. In agricultural practice, TB can be applied either before or after the rice field has been flooded. This experiment was designed to determine whether this would affect the dechlorination of TB and the persistence of TB and DTB. A soil suspension was prepared by adding 500 g of field soil and 1500 ml distilled water to a 2-L flask. The mixture was stirred and then incubated at room temperature (23-25 C) in the dark for 40 days. This suspension was stirred for 2 hs, and then 400 g of suspension was transferred into each of two 1-L beakers. For the pre-flood treatments, TB (10% ethanol solution) was added to one of the two beakers at a final concentration of 100 µg/g, mixed and then 80 ml of suspension (approximately 93 g) was dispensed into each of four 140 cc plastic

Monoject syringes (Sherwood-Davis & Geck, St. Louis, Mo.). For the after-flood treatment, suspension in the other beaker was mixed and 80 ml was dispensed into each of the four 140 cc plastic syringes. These syringes were then incubated at room temperature in the dark for 30 h to let the soil in suspension settle. TB was then added into the water phase of each syringe in an amount that would give a final concentration of 100 $\mu\text{g/g}$ based on the total weight of suspension (approximately 93.0 g) in each syringe. The suspensions inside the syringes did not complete settling until after 56 h of incubation. At this time two syringes from pre-flood and two syringes from the post-flood treatments were moved to a refrigerator (0-5 C). After 10 days of incubation, the water phase in each of the eight syringes was carefully transferred to a 100 ml –flask. All syringes were then frozen for 3 h at -70°C . Each of the frozen syringes was segmented into three equal sections (approximately 1.5 cm) of soil phase using a hacksaw. Ten grams was collected from each section and extracted with 10 ml of hexane. TB and DTB concentrations were quantified using GC/MS as previously described.

3.2.2.2 Oxygen availability centrifuge tube test. A suspension was prepared by mixing 200 g of soil with 600 ml of distilled water in a 1000 ml-flask covered with aluminum foil. The suspension was then pre-incubated at room temperature in the dark for 5 days. The suspension was shaken briefly each day. The pH values of the suspension changed from 6.5 to 7.0. After pre-incubation, TB was added to the suspension to a final concentration of 20 $\mu\text{g/g}$. The suspension was stirred vigorously for 6 h, and then 50 ml of suspension was dispensed into each of nine, screw-capped centrifuge tubes (Fisher Scientific, Pittsburgh, PA 15219). The oxygen availability was varied using two treatments: 1) a closed top, in which the tubes were tightly closed with

screw-caps; 2) open top, in which tubes remained open and incubated in a 1000ml-beaker. To reduce evaporation of water in the tubes, water was maintained in the bottom of the beaker and the top of the beaker was loosely covered with a sheet of paper. These beakers were incubated at room temperature (24-26 C) in the dark. To test the distribution of TB without significant microbial degradation, a control with tubes covered with screw caps were included and incubated at 5 C. The experimental design is shown in Figure 3-2. After 8 days incubation, water and soil samples were obtained from different depths. Soil settling in tubes incubated at room temperature was more complete. Five-ml water samples were obtained (with a 10-ml syringe) from 4 to 5 cm (W1), 2.6 to 3.5 cm (W2), and 0.2 to 1.2 cm (W3) above the soil/water interface. In cold incubation tubes, water samples were obtained from 5.2 to 6.0 cm (W1), 3.4 to 4.2 cm (W2), and 0.5 to 1.8 cm (W3) above the soil/water interface. After the water samples were obtained, the soil columns were frozen at -70°C for 3 h. Then the soil columns were mechanically sectioned with a hacksaw into three sections. For those incubated at room temperature, the position for each section was 0 to 1 cm (S1), 1 to 2.6 cm (S2), and 2.6 to 5.0 cm (S3) below the soil/water interface. For those incubated in the cold (5C), the position for each section was 0 to 1 cm (S1), 1 to 2.2 cm (S2), and 2.2 to 4.0 cm (S3) below the soil/water interface. One gram from each of the water and soil samples was extracted with 1 ml of hexane, then TB and DTB concentrations were quantified using GC/MS as previously described.

3.2.3 Relationship between redox potential and dechlorination of TB in a soil column.

3.3.3.1 Preparation of the soil column. The suspension used was from the same batch as used in the previous section. After the suspension was stirred vigorously for 6 h it was

dispensed into the following containers: 1) 140 cc plastic Monoject syringes for redox potential measurement with 80 ml of suspensions (Figure 3-1 A), 2) 140 cc plastic syringes with 80 ml suspension per syringe, the plunger of each syringe was withdrawn to the mark of 100 ml thus creating 20 ml of headspace to maintain sufficient oxygen above the suspension. This set of syringes was included for the analysis of TB and DTB concentrations using hexane extraction and GC/MS quantification.

3.2.3.2 Redox potential measurement. A special apparatus for measuring redox potential at different depths of soil suspensions was constructed. Platinum wire (Fisher Scientific, Pittsburgh, PA) with a diameter of 1.024 mm was cut into 1.3 cm segments and treated following the procedure described by Patrick et al. (1996). Two rows of seven holes, with diameter similar to that of the platinum wire segments were drilled along opposite sides of a 140-ml syringe (Figure 3-1 A). The distance between the adjacent segments would be, from the top of the syringe to the bottom, 1, 2, 1, 0.5, 1, and 1 cm, respectively. A platinum wire segment was placed into each hole and the hole was sealed with Epoxy (Devcon, Riviera, Beach, FL33404) to prevent any leakage of the aqueous solution. The redox apparatus created a soil/water interface between the third and the fourth platinum segments after being filled with 80 ml of suspension. The top three platinum segments were used to measure the redox potential of the water phase (approximately 3.7, 2.7, and 0.7 cm above the soil/water interface) while the bottom four segments corresponded to the redox potential in the soil column approximately 0.3, 0.8, 1.8, and 2.8 cm below the soil/water interface. Redox potential was measured by connecting each platinum segment with an alligator clip to a pH meter (ORION model 920A). A saturated calomel reference electrode was used to complete the cell. The

direct reading (mV) was transformed into redox potential relative to hydrogen gas (Eh) by adding a correction factor of +245 mV (Patrick et al. 1996).

The consistency of the redox apparatus was tested by comparing the redox potential on the two sets of platinum segments on opposite sides of the syringe and the redox reading on two separate apparatus, each containing the same suspension. The experiment was conducted twice.

3.2.3.3 Parallel test to relate dechlorination of TB to redox potential profiles in a soil column. For the determination of dechlorination of TB at different depths of a soil column, two 140 cc plastic syringes were used to prepare soil columns as the pre-flood treatment described before (TB added and mixed). After incubation at room temperature in the dark for 14 days, the water phase was removed at three different depths (corresponding to 10, 20, and 30 mm above the soil/water interface), and the soil columns were frozen at -70°C for 3 h and then sectioned into six segments, corresponding to approximately 2.5, 7.5, 12.5, 17.5, 22.5, 27.5 mm below the soil/water interface. Five grams of sample from each segment was extracted with 5 ml of hexane and subjected to GC/MS analysis as previously described.

3.2.4 Effect of starch and anaerobiosis on dechlorination of TB in an enriched suspension. This experiment was to determine if the addition of starch, which would reduce oxygen concentration through microbial decomposition, or the exclusion of air would have any effect on the redox potential and dechlorination of TB in an activated (enriched) soil suspension. The enriched suspension was prepared by mixing 400 g of

A



B

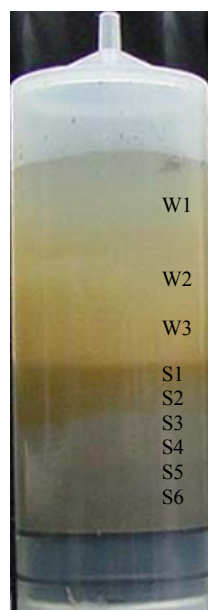


Figure 3-1. Experimental soil columns and the redox potential measurement apparatus. A. Apparatus used to measure the redox potential of a soil column. On top of the syringe is a saturated calomel electrode, which is connected to a pH meter together with one of the platinum segments on two sides of the syringe. The arrow indicates the soil/water interface, B. Soil columns used for the quantification of thiobencarb and dechlorinated thiobencarb (after 14 days of incubation). The position of water samples (W1, W2, and W3) and soil samples (S1, S2, S3, S4, S5, and S6) are indicated.

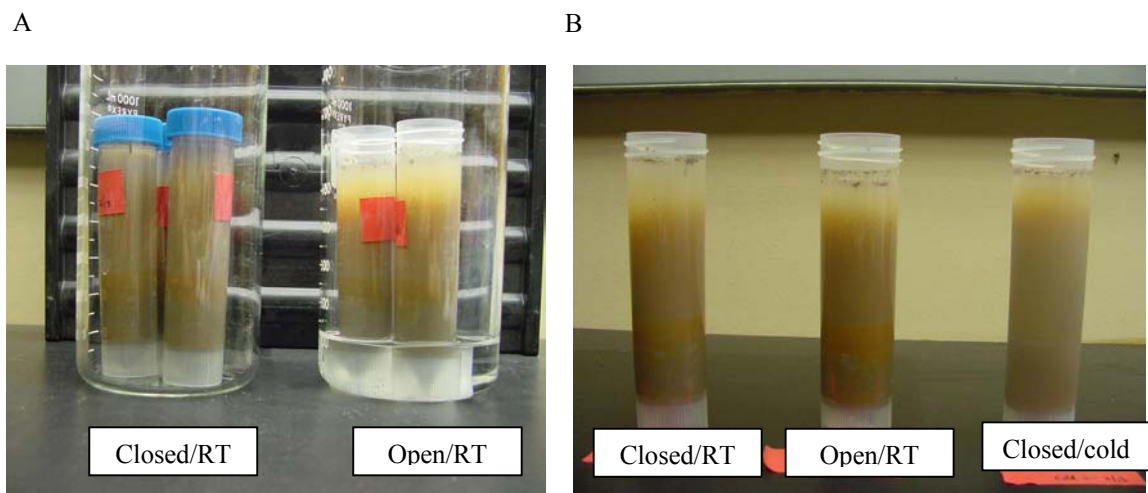


Figure 3-2. Treatments for the determination of thiobencarb and dechlorinated thiobencarb distributions in a centrifuge tube test. A. Tubes treated with closed or open caps inside a beaker after 8 days of incubation at room temperature in the dark. B. Tubes of soil suspension after treatment with closed top (Closed) or open top (Open), and incubated at room temperature (RT)/or at 5 C (Cold).

DPS-conductive soil in 600 ml of water and then incubating at room temperature for 5 days. TB (10 µg/g final concentration) was then added to the suspension and incubated for an additional 2.5 months. GC/MS analysis of the samples indicated TB was completely transformed into DTB, suggesting dechlorinating organisms were active (enriched). Two treatments were included: suspension plus 0.1% soluble starch (Difco Laboratories, Detroit, MI) and suspension without soluble starch. TB was added into the suspension at 10 µg/g (final concentration), mixed and then distributed into 140 cc-syringes at 80 ml/syringe. Each treatment was duplicated. These syringes were incubated in the dark for 18 days at room temperature. One water sample was collected from each syringe using a 5-ml syringe. After removal of residual water from the syringe, the soil phase was frozen and sectioned into three equal sections. A duplicate set of syringes was used for redox potential measurement following the procedures described previously. The only difference was that the space between the reference electrode and the mouth of the syringe was sealed with Vaseline to prevent air circulation.

3.3 Results

3.3.1 Determining TB and DTB concentrations in partitioned soil columns.

3.3.1.1 Effects of timing of application of TB on DTB formation in a syringe test.

Only a very small amount of TB was dechlorinated while the majority of TB still remained after 8 days of incubation (Table 3-1). When concentrations of the residual TB were compared, distribution of TB in the soil column depended largely on timing of application and position in the column. For the treatments in which TB was added and mixed before the precipitation of the suspension, the majority of TB was located in the bottom two soil sections (with a thickness of approximately 3 cm), regardless of whether

the syringes were incubated at room temperature or at 5 C. TB concentrations in the water and soil phases were generally higher when incubated at 5 C than when incubated at room temperature, especially in the bottom 1.5 cm soil phase. In the bottom soil section, TB concentration for the cold treatment was 114.8 µg/g while that of the room temperature treatment was 63.0 µg/g. Degradation and transformation in the cold treatment were much slower. In the treatments in which TB was applied directly into the water phase before the suspension had completely precipitated (after 30 h incubation), large amounts of TB were detected in the water phase and the top 3.0 cm soil phase. No TB was present in the bottom 1.5 cm soil phase. When the room temperature treatment and cold treatment soil phases were compared, the top 1.5 cm section of soil in the cold treatment had much higher TB concentrations than that in room temperature treatment (186.9 µg/ml vs. 56.6 µg/g). The difference in the middle 1.5 cm section for those two treatments was less (188.1 µg/g vs. 134.4 µg/g). No TB was detected in the bottom 1.5 cm soil phase of either treatment, suggesting TB had little vertical mobility in the soil.

DTB was only detected in samples incubated at room temperature (Table 3-1). In the treatment where TB was added into soil and incubated at room temperature, a significant amount of DTB (9.6 µg/g) accumulated in the bottom 1.5 cm soil phase. Only a small amount of DTB accumulated in the middle soil (0.6 µg/g) and top 1.5 cm soil sections (0.2 µg/g). No DTB was detected in the water phase. In the treatment where TB was added into the water phase after the suspension had settled, only trace amounts of DTB were detected in the water phase. No DTB was detected in the bottom section. In all the cold treatments, no DTB was detected regardless of whether TB was pre-mixed or post-mixed.

Table 3-1. Effect of cold treatment and timing of thiobencarb (TB) application on the distribution of TB and dechlorinated TB (DTB) in soil columns after 8 days of incubation

Treatment		TB ($\mu\text{g/g}$) ^{wz}				DTB ($\mu\text{g/g}$) ^{wz}			
Temp ^x	Time ^y	W	S1	S2	S3	W	S1	S2	S3
5 C	After	NA	186.9 a	188.1 a	0.1 b	0	0	0	0
23-25 C	After	32.4 b	56.6 b	134.4 a	0 b	0.3	0.2	0.2	0
5 C	Before	4.3 c	11.4 bc	95.1 ab	114.8 a	0	0	0	0
23-25 C	Before	1.8 c	11.4 bc	92.6 a	63.0 ab	0 b	0.2 b	0.6 b	9.6a

^w. Means followed by the same letters within either TB or DTB in each row are not significantly different (LSD, $P=0.05$).

^x Incubation temperature. The columns were stored in the refrigerator (5 C) or incubated at room temperature (23-25 C).

^y TB applied after settling of soil particles by adding into the water phase (After) or TB was applied (Before) the suspension was stirred and dispensed into the syringe.

^z Water phase (W); S1, S2, and S3 represent three soil sections from top, middle, and bottom of the soil column, respectively.

3.3.1.2 Oxygen availability centrifuge tube test. Among the three treatments, the cold treatment produced a more narrow soil phase than the room temperature treatments; more soil stayed in suspension. After 8 days of incubation in centrifuge tubes, residual TB concentrations were compared among the three treatments, the cold treatment had higher TB in the water and soil phases than those incubated at room temperature (Figure 3-3 A). The water phase contained very small amounts of TB, regardless of position. The TB distribution curves of the tubes incubated at room temperature and the closed cap and open cap treatments were very similar. Among the three soil sections, the middle section had more TB than the sections below or above it. The upper section had the lowest TB among the three soil sections, and the TB concentration was much lower than the same position in the cold treatment, suggesting that there was more TB degraded in the upper section than in the middle and bottom sections.

DTB concentrations were only detected in the lower soil phases and in low concentrations when the soil columns were incubated at room temperature (Figure 3-3 B). When DTB distributions were compared among the three treatments, a significant amount of DTB was detected in the bottom soil column sections for the room temperature treatment, regardless of closed or open treatments. No DTB was detected in the water or soil phases for the cold treatment. As this was still in the early stage of dechlorination of TB (8 days after incubation), there was less than 0.5 µg/ml of DTB in any section. The bottom and middle sections had similar DTB concentrations for either closed or open treatment. The upper soil sections had only trace amounts of DTB. Treatments with the tube cap off appeared to have slightly higher DTB production than tubes with closed caps, but the difference was not significant.

3.3.2 Relationship between redox potential and dechlorination of TB in a soil column.

3.3.2.1 Consistency of the redox measurement apparatus. At the beginning of the experiment and after 14 days incubation, the Eh readings were almost identical for the two opposing sets of Pt segments (Figure 3-4). The Eh readings from two different apparatuses were very similar (data not shown). This result suggests that the redox potential measurement apparatus provided a reproducible way to estimate the distribution of redox potential in a soil column.

3.3.2.2 Parallel test to relate dechlorination of TB to redox potential profiles in a soil column The distribution of combined TB and DTB concentrations at different depths in the water and soil phases of a soil column are shown in Figure 3-5. There was

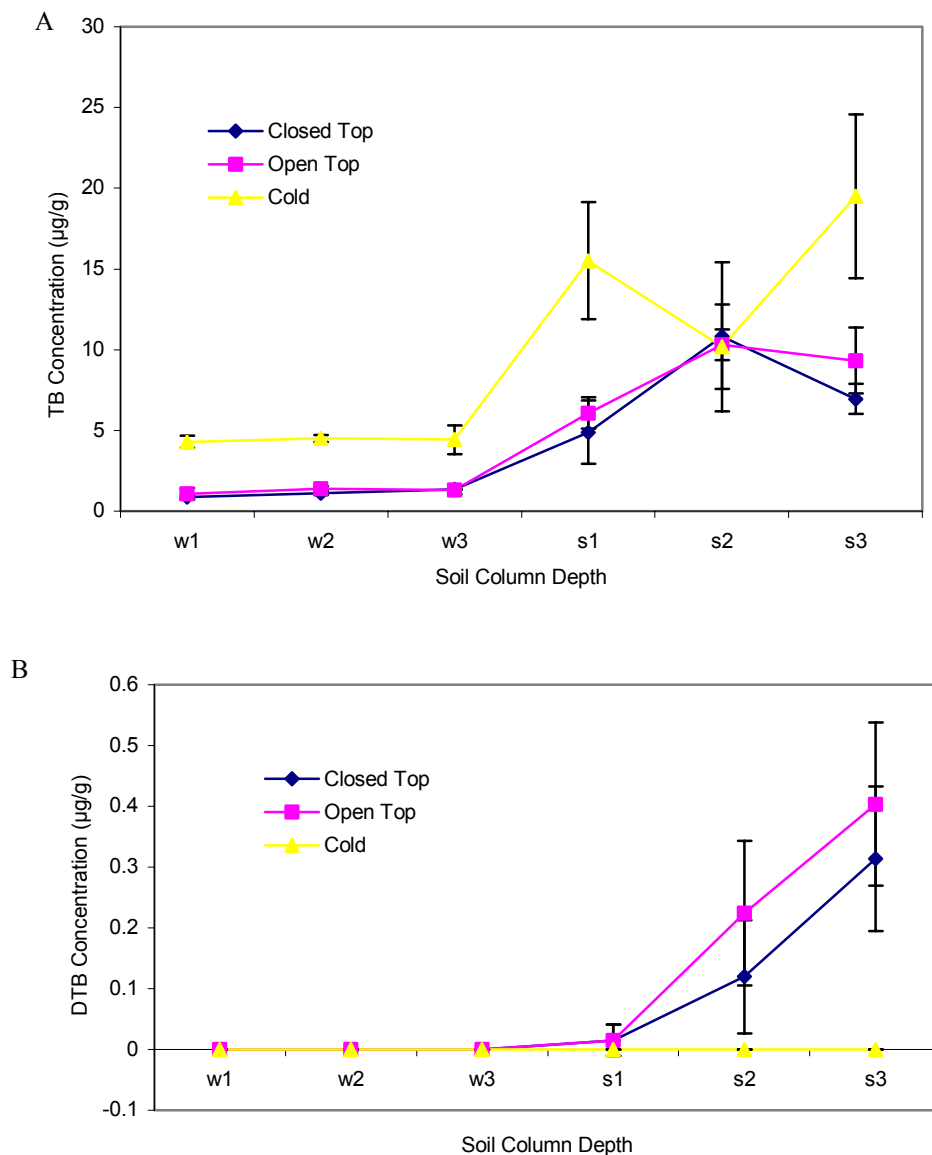


Figure 3-3. The effect of oxygen availability on the distribution of thiobencarb (TB) (A) or dechlorinated thiobencarb (DTB) (B) in soil columns prepared with 50 ml centrifuge tubes as containers. Cold: tubes incubated at 5 C. Closed Top: tubes incubated at 23-25 C with caps tightened. Open Top: tubes incubated at 23-25 C with the caps removed. Water samples were obtained from 4 to 5 cm (W1), 2.6 to 3.5 cm (W2), and 0.2 to 1.2 cm (W3) above the soil/water interface. Soil samples for each section were obtained from 0 to 1 cm (S1), 1 to 2.6 cm (S2), and 2.6 to 5.0 cm (S3) below the soil/water interface. For the tubes under cold incubation, water samples were obtained from 5.2 to 6.0 cm (W1), 3.4 to 4.2 cm (W2), and 0.5 to 1.8 cm (W3) above the soil/water interface. Soil sample was obtained from 0 to 1 cm (S1), 1 to 2.2 cm (S2), and 2.2 to 4.0 cm (S3) below the soil/water interface. Error bars represent standard deviations.

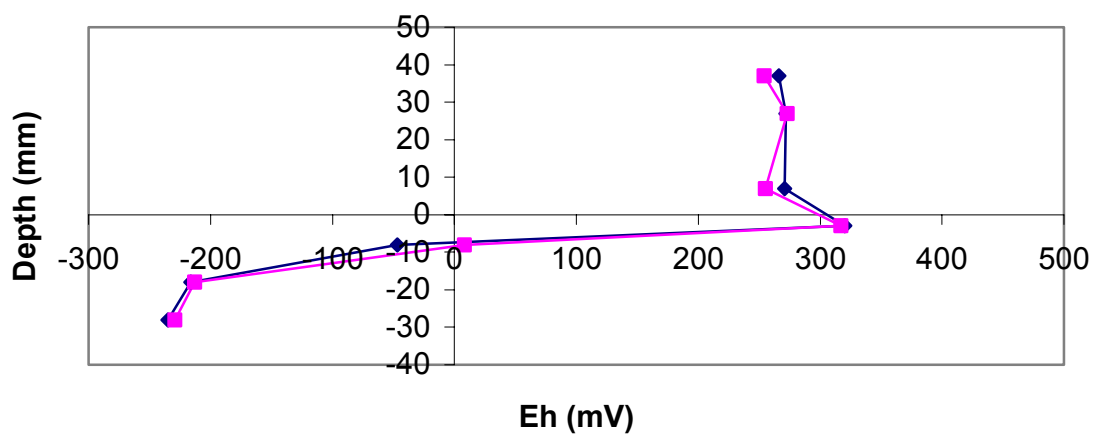
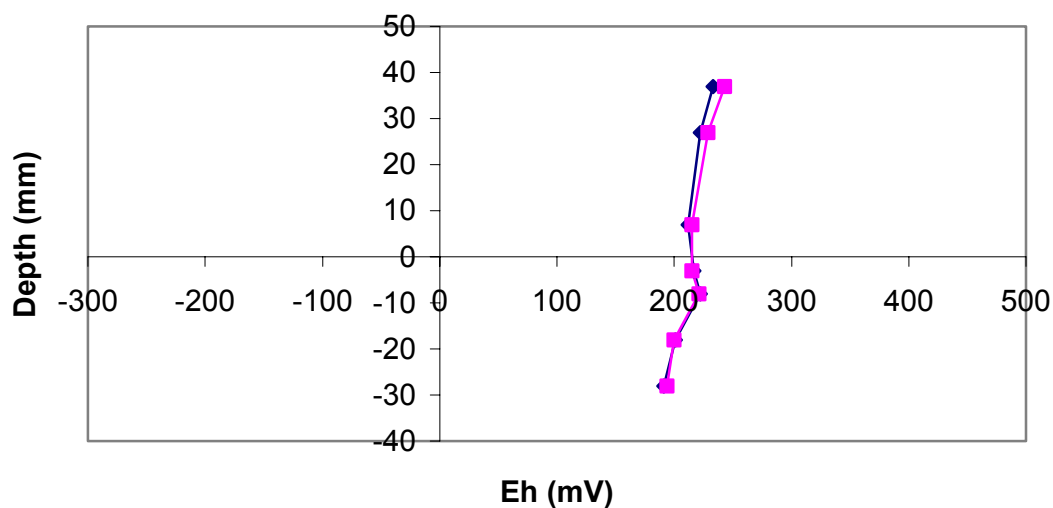


Figure 3-4. The consistency of the redox measurement apparatus was checked by comparing the readings of each pair of platinum segments on opposite sides of the syringe. The change of redox potential at the start (top) and 14 days (bottom) was reflected by two sets of platinum wires on opposite sides of a syringe containing Crowley silt loam soil suspended in water.

more TB and DTB located in the soil segment next to the bottom segment than any other positions. Some of the DTB and the residual TB were located in the last four segments, while the water phase contained low amounts of either TB or DTB. Thus, most of the dechlorination activity apparently occurred in the bottom three segments.

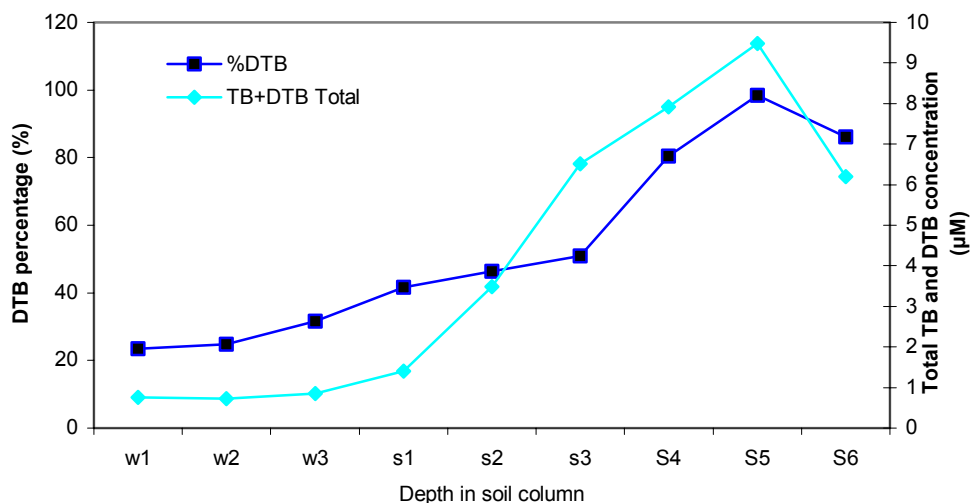


Figure 3-5. The distribution of thiobencarb (TB) and dechlorinated thiobencarb (DTB) at different depths of water and soil phases inside 140-ml syringes after 14 days of incubation. The solid line represents the percentage of DTB while the dotted line represents the total concentration of DTB and the residual concentrations of TB concentration combined (μM). The symbols w1, w2, and w3 represent water samples collected at 10, 20, and 30 mm above the soil/water interface, respectively. The symbols s1, s2, s3, s4, s5, and s6 represent soil samples collected at approximately 2.5, 7.5, 12.5, 17.5, 22.5, and 27.5 mm below the soil/water interface, respectively (as shown in Figure 3-1).

The redox profiles of the parallel soil columns (containing TB and DTB) are depicted in Figure 3-6. At 0 h of incubation, all positions in the soil column show Eh values that were equal to or higher than + 200 mV. The platinum segments imbedded in soil portions of the soil column showed lower Eh values (more negative) than those in the water phases after 1 day of incubation. The lowest Eh value in the soil was –100 mV. Except

for the platinum segments, which were imbedded 5 mm below the floodwater/soil interface, all platinum segments imbedded in lower positions showed negative Eh values 5 days after incubation. The values of Eh decreased as the soil went deeper. The lowest Eh value (−210 mV) was located at the lowest platinum segments imbedded in the soil column. The platinum segments located at the soil position next to the bottom had Eh of −160 mV. The decreased of Eh values for these two positions continued until 14 days after incubation. By this time the lowest segment had an Eh value of −232 mV while the position next to the bottom had an Eh of −215 mV.

When the distribution of DTB and the redox potential profiles in a soil column were taken into consideration (Figure 3-7), the peak of DTB concentration was at the soil position corresponding to an Eh of approximately −215 mV after incubation for 14 days.

3.3.3 Effect of starch and anaerobiosis on dechlorination of TB in an enriched

suspension. After 18 days of incubation, the treatment with soluble starch added had a lower concentration of DTB than treatments without starch throughout the soil column (Figure 3-8). However, in the water phase, the DTB concentration was higher for the starch treatment than the treatments without starch, which had no DTB.

The treatments without additional starch had an Eh of −200 mV or less in the water phase and soil phase (Figure 3-9). However, in the soil treated with soluble starch, Eh decreased dramatically to −350 mV in the soil after 1 day of incubation. A significant amount of gas was generated in the syringes treated with soluble starch, pushing water out of the open top of the syringes. The water level in the starch treatments was lowered and the top two Pt segments were exposed to the air, thus no Eh values were obtained thereafter. Eh then increased gradually, reaching −200 mV after 18 days incubation.

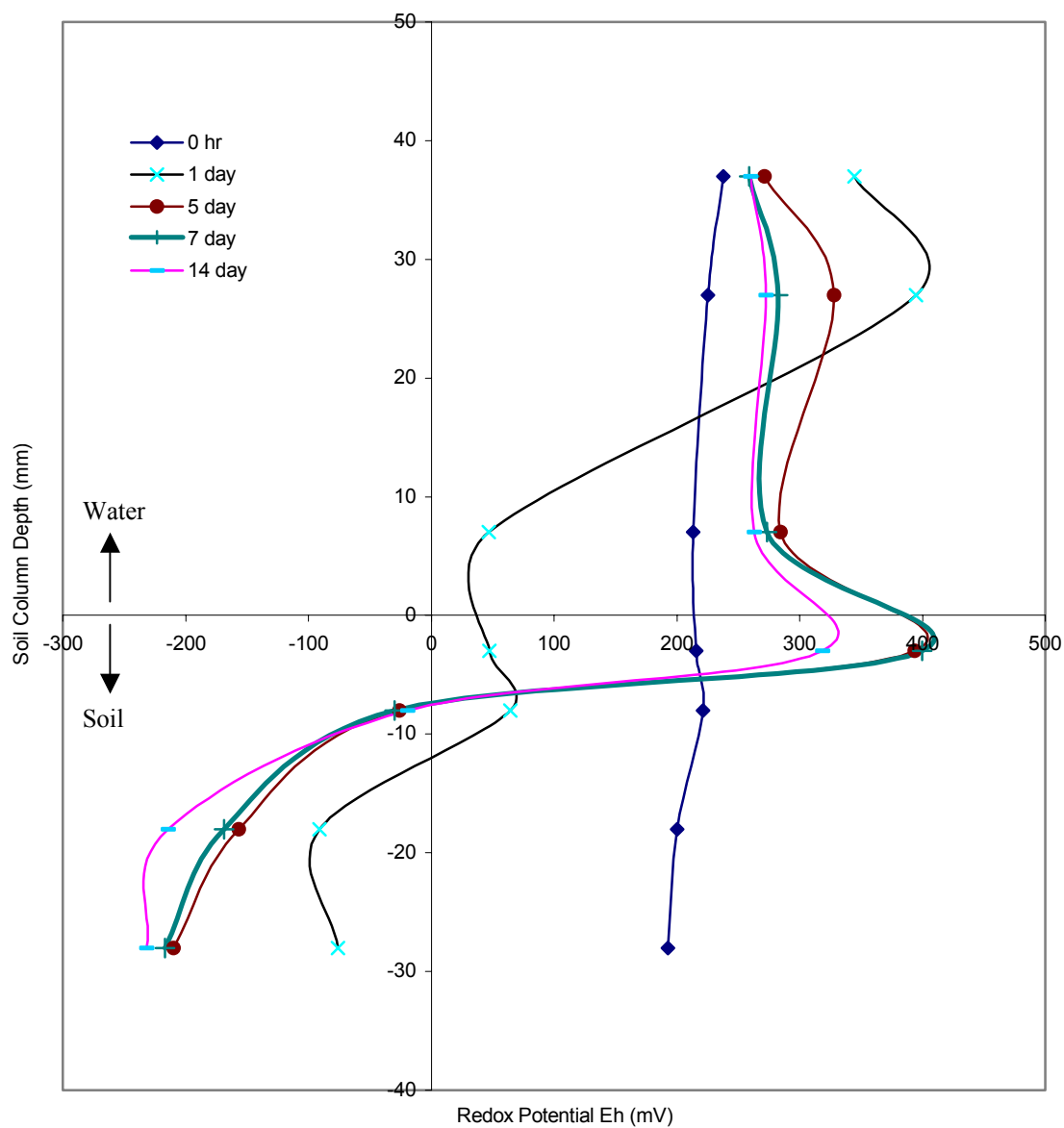


Figure 3-6. Redox potential profiles of a soil column after 0 to 14 days of incubation. The soil column was prepared with a suspension made by mixing conductive soil and water in a 140-cc plastic syringe as described in the text.

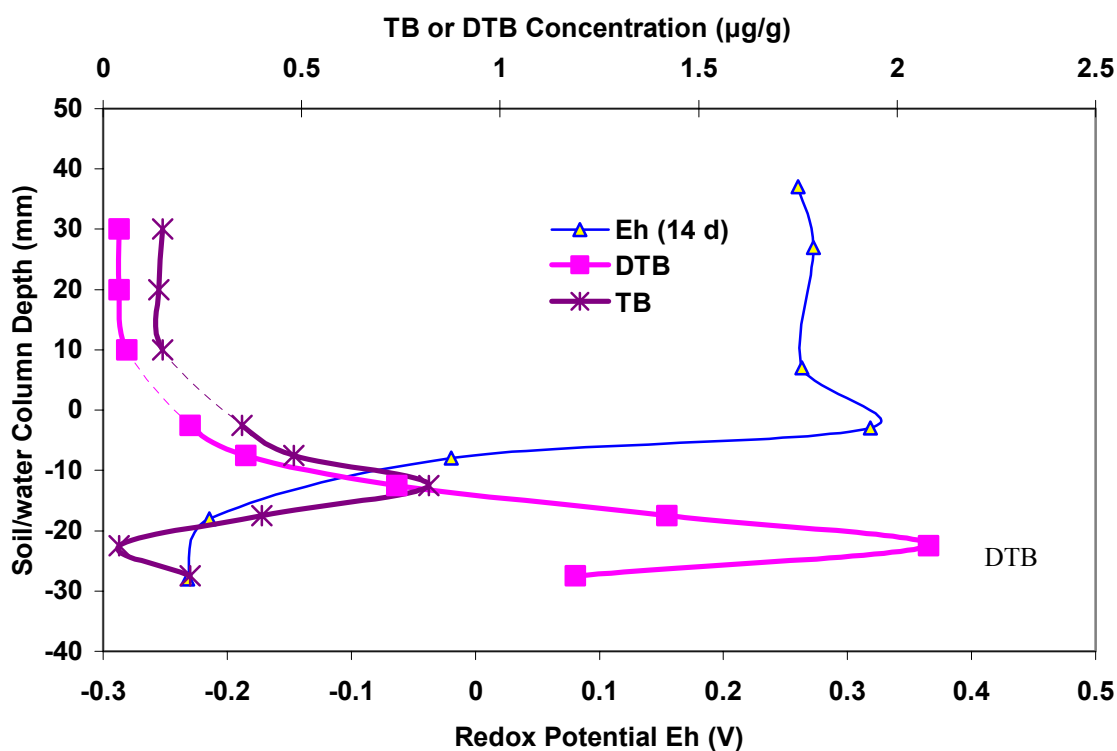


Figure 3-7. The relationship between redox potential and dechlorination of thiobencarb (TB) in a soil column. The concentrations of dechlorinated thiobencarb (DTB), and the residual TB (in $\mu\text{g/g}$) after 14 days of incubation were plotted together with the redox profiles for a similar soil suspension column after 14 days of incubation. The depth of 0 represents the soil/water interface.

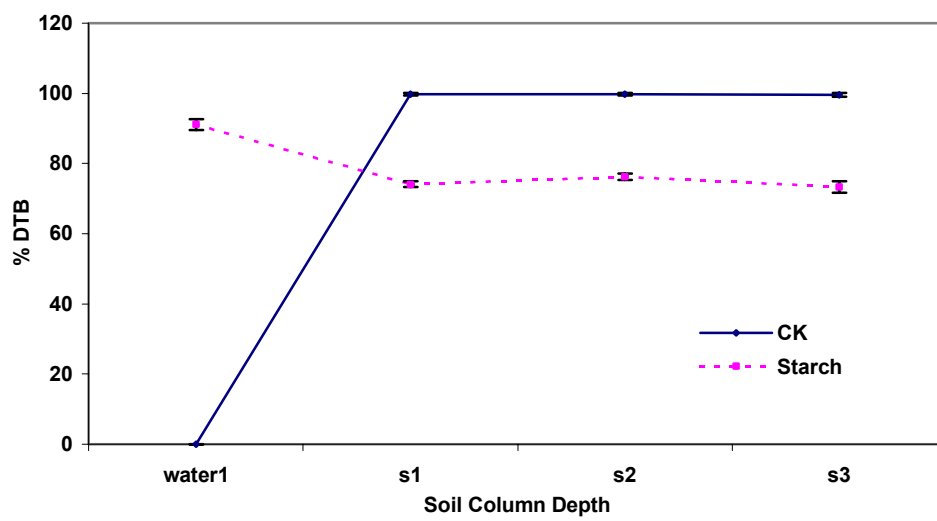


Figure 3-8. The percentage of thiobencarb being dechlorinated at different depths of a soil column as affected by the addition of 0.1% soluble starch. Error bars represent standard deviations.

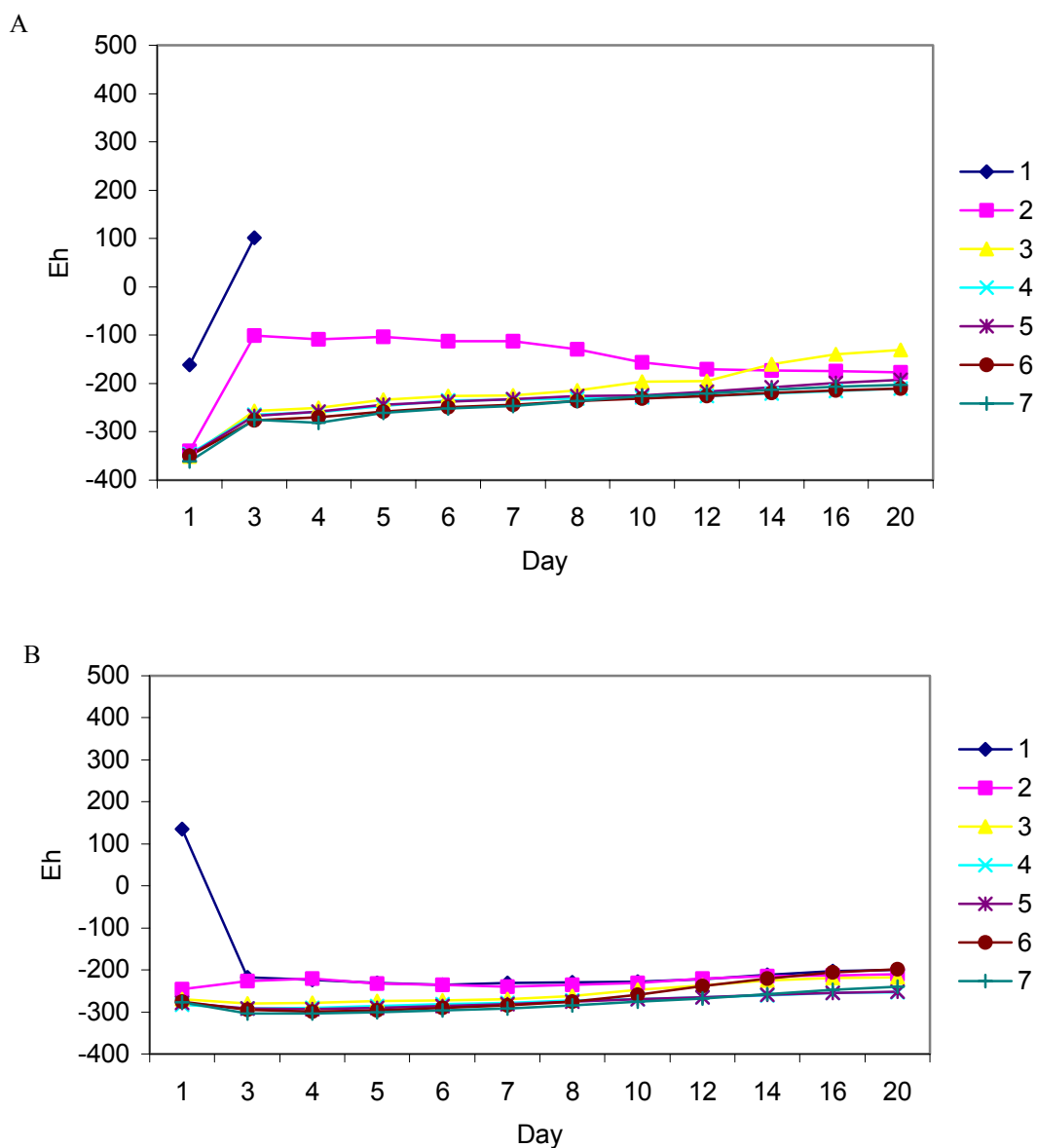


Figure 3-9. Changes in redox potential (Eh) over time at different depths of a soil column with (A) and without (B) 0.1% soluble starch added. The numbers on the right of each figure indicates the platinum segments from top to the bottom of the measurement apparatus, 1 represents the Pt electrode in the water phase while 2, 3, 4, 5, 6, and 7 represent the Pt electrodes at increasing depths in the soil column.

3.4 Discussion

To estimate the relationship between redox condition of a rice soil and reductive dechlorination of TB, a special apparatus capable of measuring redox potential at different depths and over different times was constructed. This apparatus was improved from previous versions (Banker et al. 1995; Patrick and Delaune 1972) because 1) the soil column was kept stationary throughout the test period; no vertical movement of soil or water phases was involved, thereby preventing mechanical movement of TB and DTB inside the column; 2) two sets of Pt sensors served as replicates on opposite sides of the syringe to increase accuracy; 3) contact was continuous with soil or water throughout the test period, thus avoiding the problem of slow equilibration when redox potentials were low; 4) multiple Pt electrodes were spaced throughout the water and soil phases, thus giving redox readings in the water and soil column at different depths. The redox potential profiles of the soil suspension were repeatable. However, a problem with the apparatus was that the space between adjacent Pt electrodes was too large for some zones, especially around the floodwater/soil interface. In a typical rice field soil, there is only a 2-3 mm- zone below the floodwater/soil interface where oxygen can penetrate (Liesack et al. 2000). The area beneath this zone would be anoxic. Seven Pt segments were put into each side of the apparatus. Three were in contact with the water phase and four were in contact with the soil phase. Although one Pt segment was in the zone that was 5 mm below the soil/water interface of the soil column, there may not have been enough coverage of the oxidized/reduced transition zone of flooded soil. An earlier study indicated the transition zone occurred within an 8 mm zone (Patrick and Delaune 1972). This transition zone could be even thinner as oxygen was depleted from 140 μM at the

floodwater/soil interface to nondetectable amounts at a depth of 1.6 to 2.2 mm and below after 7 days of incubation at 30 C (Ludemann et al. 2000).

However, failure to pinpoint a precise location for the transition zone for the soil column did not seem to have a significant effect on our ability to quantify the relationship between redox conditions and dechlorination of TB. The optimized zone for reductive dechlorination of TB occurred approximately 20 mm below the soil/water interface, the zones above and below the optimum zone had lower DTB concentrations. Redox measurements showed that these zones quickly became reduced after soil in the suspension settled. Values of Eh in these zones decreased to around -100 mV after one day of incubation and decreased to around -200 mV after 5 days incubation. However, DTB was not detected after 8 days incubation, suggesting that the reduced (anaerobic) conditions in the zones favorable for dechlorination needed to be sustained for some time for significant amounts of DTB to form. This observation also reflects those observed in field conditions, where DPS was observed 2 weeks after flooding and application of TB (Groth et al 1999). Other zones in the column, including all the water phases and the top 10 mm of the soil zone, showed a low dechlorination rate. The Eh values for these zones remained above -100 mV throughout the experiment. The Eh values that supported or did not support the reductive dechlorination of TB were very similar to those reported by Moon and Kuwatsuka (1985b).

Zones of DTB accumulation would be the most probable location to isolate the dechlorinating organisms. However, the microbial activities in the zones of optimized dechlorination remains to be studied. During the formation of a soil column from a suspension, many chemical and biological reactions are taking place, such as oxidation,

fermentation, sulfate reduction, or methanogenesis. The organic materials in the soil are being broken down by microbial activity. During this process microbes consume oxygen and produce reducing equivalents. Redox potentials fluctuate due to the changes of oxygen and reducing equivalents. Methanogenic activity becomes significant once the Eh reaches a critical point of -150 mV or lower in flooded rice field soils (Banker et al. 1995). Whether methanogenic bacteria were involved in the reductive dechlorination of TB is not clear.

Due to the different absorption properties of soil components to TB (Mabury et al. 1996), TB was not evenly distributed throughout the soil column. TB, as with other pesticides, is bound reversibly into the bottom sediment by the process of adsorption. Among the soil components; sand, silt, clay, and organic matter, the organic matter has proven to be the most important for absorption (Mabury et al. 1996). Although TB was not considered as strongly bound to sediment as other rice field pesticides such as DDT and mirex, its absorption to sediment was much stronger than those of MCPA and molinate. Because of this previous research our suspensions were vigorously stirred for several hours before dispensing the suspension into the syringes. However, different soil components were precipitated at different rates as the suspension settled. The bottom of the column was rich in sand, the next zones close to the bottom were rich in silt then clay. This would be similar to stratification in rice fields cultivated in water before seeding. This would probably explain why the amounts of TB and DTB were higher in the zone close to the bottom. However, the TB dechlorination rate was also lower in the zone next to the bottom than in the bottom zone. Both of these zones had very similar redox potentials throughout the experiment. Factors other than redox potential might have

contributed to the different dechlorination rates. These factors may include hydrogen and methane formation, and nutrient distributions (Patrick & Henderson 1981; Reddy et al. 1987; 1990). These factors remain to be studied.

For the isolation of potential dechlorinating microorganisms, the zone with low redox potential and high dechlorination of TB may be a good source. This study demonstrated that this was a zone where dechlorination of TB had been optimized, presumably because of more dechlorinating organisms

The apparatus (140 cc plastic syringe) could be an ideal test system for determining if a microorganism can dechlorinate TB since the system can be sterilized and sealed. However, a non-dechlorinating bacterium may need to be added to produce anaerobic conditions. The Eh values in the column and in field soil were similar for dechlorination of TB.

Chapter 4

Microorganisms Implicated in the Reductive Dechlorination of Thiobencarb in South Louisiana Rice Field Soil

4.1 Introduction

Halogenated aromatic compounds are generally resistant to biodegradation because the halogen is both larger and more resistant to electron withdrawal than hydrogen. As a result, these halogens can have detrimental steric and electronic effects on the ability of enzymes to catalyze the required degradation reactions (Crooks & Copley 1993). Reductive dehalogenation is defined by the removal of a halogen atom from a molecule with concurrent addition of electrons to the molecule (Mohn & Tiedje 1992). It is typically biologically dependent, but it is not clear that the activity is biologically catalyzed. In all reported examples of biologically catalyzed reductive dehalogenation, the halogen atoms are released as halide anions. Reductive dehalogenation is mainly known to occur under anaerobic conditions and is the initial step in anaerobic biodegradation of most aryl halides. Anaerobic (Bouchard et al. 1996; DeWeerd et al. 1990; Utkin et al. 1994), facultative anaerobic (Cole et al. 1994; Tsuchiya & Yamaha 1984), and aerobic bacteria (Enzien et al. 1994; Schenzle et al. 1999; van den Tweel et al. 1987) were reported to be capable of reductive dehalogenation, either through whole cell activity or by their enzymes. A white mold fungus, *Phanerochete chrysosporium*, also used reductive dehalogenation in some steps of degradation of halogenated aromatic compounds (Reddy et al. 1998). In biologically dependent reductive dehalogenation, there are two important phases: the acclimation phase and enrichment phase. Acclimation is used to describe the initial period of incubation during which reductive dehalogenation is not detectable, and is sometimes referred to as the lag

time. This phase appears to be more commonly associated with aryl than alkyl reductive dehalogenation (Mohn & Tiedje 1992). The enrichment phase is noted for the increase in dehalogenation rates and decrease in acclimation periods in transfers from primary cultures. An enrichment of reductive dehalogenation would indicate that organisms involved might have benefited from the activity. The possible benefits for the dehalogenation organisms include obtaining energy from the process, which has been termed dehalorespiration (Holliger et al. 1999), or using the dehalogenated product as a carbon or energy source (van den Tweel et al. 1987). Reductive dehalogenation can be affected by many factors. Electron acceptors are frequently believed to be the limiting resources for anaerobic communities and a major determinant of the species composition of communities.

Oxygen was believed to be inhibitory to reductive dehalogenation, based on the observation that reductive dehalogenation typically occurs in natural environments only if they are anaerobic and usually require low redox potential. Although oxygen, nitrate, and sulfate most often inhibit dehalogenation by anaerobic communities, the nature of such inhibition varies under different biological and chemical conditions (Mohn and Tiedje 1992).

Many studies reported that dehalogenation of aromatic compounds involved co-metabolic or abiotic reductive dehalogenation (Holliger et al. 1999). In most cases, the dehalogenation appeared to be mediated in cell-free systems by heat-stable compounds at slow rates. Tetrapyrrole-cofactors, such as corrinoids, iron porphyrins, and coenzyme F430 in aqueous solution, are known to be capable of reductive dehalogenation of polychlorinated ethylenes and benzenes (Gantzer & Wackett 1991). However, the rates

of dechlorination decreased dramatically with decreasing chlorine content in the compounds. Monochlorobenzenes were not dechlorinated in the solutions (Gantzer & Wackett 1991).

The reductive dechlorination of the herbicide TB in rice field soil was first reported in Japan in 1981 (Ishikawa et al. 1980) and later in the US (Groth et al. 1999). Rice plants exposed to DTB showed phytotoxic symptoms such as dwarfing and excessive tillering. This disorder was termed delayed phytotoxicity syndrome (DPS) (Groth & Sanders 1996). A biological cause of DPS was suggested because damaged plants were in irregularly shaped areas and unevenly distributed within fields (Groth et al. 1999). Dechlorination appeared proportional to the ratio of conducive/nonconductive soil in the mixtures, and repeated application of TB shortened the lag period for dechlorination (Tatsuyama et al. 1981; Moon & Kuwatsuka, 1984, 1985a). Furthermore, dechlorination was inhibited by application of certain antibiotics (Moon & Kuwatsuka, 1985a) or fungicides (Groth et al. 1999). Dechlorination of TB peaked at 25-30 C and pH 7 (Moon & Kuwatsuka 1985a), which are ideal conditions for the growth of many microorganisms.

Organisms responsible for the reductive dechlorination of TB have not been isolated. Although a facultative bacterium had been suggested as responsible for DPS in Japan, certain groups of fungi such as *Trichoderma* sp. and *Phanerochaete chrysosporium* were shown to be involved in DPS in Louisiana (Groth & Sanders 1996). The objectives of this study were 1) to characterize the pattern of reductive dechlorination of TB in rice field soil; 2) to test the effects of different groups of organisms on dechlorination of TB under

greenhouse conditions and a more defined flask system; and 3) to identify possible microorganisms involved in dechlorination of TB.

4.2 Materials and Methods

4.2.1 Soil, soil suspension, and soil extract. A DPS-conductive soil was collected from the South Farm Unit of the Louisiana State University Rice Research Station, Crowley, Louisiana. Soil type was a Crowley silt loam, which was identified as a conducive soil for dechlorination of TB (Bollich et al. 1996). Physico-chemical properties of the soil were pH 5.6, organic matter 1.18%, and extractable nutrients (mg/kg): P (67-75), Na (66-68), K (62-69), Ca (1037-1100), Mg (229-238), Zn (4.91-5.91), S (9.19-9.3), As (4.64-4.74), and Fe (183-185.7). Soil was collected during the early spring when the field was dry, then dried further in the greenhouse. The soil was then ground into powder with a soil grinder and stored at 5 C until used.

Unless specified, suspensions were prepared by mixing 200 g of soil with 600 ml of sterile, distilled water in a 1000-ml flask, covering with aluminum foil, and incubating at room temperature (approximately 25 C) in the dark for 7-8 days.

Procedures for preparing the soil extract were modified from those of Moon and Kuwatsuka (1985a). Soil extract was prepared by mixing 500 g of South Farm soil with 1000 ml of distilled water in a 2-L flask, shaking for 1 h and then autoclaving (15 psi, 30 minutes). The flask was then shaken for another hour, the liquid phase was poured into centrifuge bottles and centrifuged at 5,000 g, the liquid phase was poured into a beaker, filtered through #1 filter paper (Whatman Ltd., England), kept overnight, and then filter-sterilized through a membrane filter (0.45 μ M). The resulting liquid was adjusted to 1000

ml and potassium phosphate was added to a concentration of 0.02 M. A sodium hydroxide solution (1N) was used to adjust the pH to 7.0. The liquid was autoclaved.

4.2.2 Effects of sterilization of conducive soil on DPS. To test if dechlorination of TB was biologically dependent, a greenhouse experiment was set up to test the effect of autoclaving on a DPS-conductive soil. The design of this test was a randomized complete design with nine treatments and four replications. DPS-conductive soil was used for six treatments: autoclaved soil without Bolero (ASF 0); autoclaved soil with Bolero 8 EC (ASF 8 EC); autoclaved soil with Bolero 10 G (ASF 8 EC); nonautoclaved soil without Bolero (SF 0); nonautoclaved soil with Bolero 8 EC (SF 8EC), nonautoclaved soil with Bolero 10G (SF10G). A Crowley silt loam soil collected from the LSU Rice Research Station North Farm was used as the conducive soil control with three treatments: autoclaved soil without Bolero (NF 0); nonautoclaved soil with Bolero 10 G (NF 10G) or without Bolero (NF 0).

Soil was placed into autoclavable bags at 1.2 kg/bag and then autoclaved (15 psi, 60 min.) on two consecutive days. The bag of soil was placed in a plastic pot (with diameter of 18 cm). The top of the bag was opened, and filled with 900 ml tap water. The soil and water were then thoroughly mixed with a wooden garden stake. Bolero 8 EC or 10 G was applied at 4.5 kg a.i./ha based on the surface area of the pots. Rice seedlings (cv. Bengal), grown in a flat with sterilized, non-conductive soil for 7 to 10 days in the greenhouse, were transplanted at two plants/pot. Water level was maintained to the top of the pot. The test was conducted in the greenhouse facilities in the Rice Research Station in Crowley, LA from July to September, 1998. Rice plants were

inspected for the presence of DPS periodically. Plant height, tiller numbers and grain weight were measured when the plants matured. The experiment was conducted twice.

4.2.3 The acclimatization of soil microorganisms for dechlorination of TB in different types of containers. This test was to determine: 1) the effect of previous application of TB on the dechlorination of subsequent applications of TB; 2) the effect of previous exposure to DTB on dechlorination of TB; and 3) the effect of container types on dechlorination as affected by air availability to the soil suspension. Three types of containers were used: 1-L flask (KIMAX), 1-L media bottle (Pyrex brand graduated laboratory bottle), and 0.5-L media bottle (Pyrex). There were two treatments for each group of containers: 1) initially treated with TB and 2) initially treated with DTB.

Two hundred grams of DPS-conductive soil (described earlier) was added to each container. The amount of sterile, distilled water used to flood the soil inside each container varied according to the size of container used: 474 ml was used for each 1-L flask, 477 ml was used for each 1-L media bottle and 286 ml was used for each of 0.5-L media bottle. TB or DTB were then added to the appropriate container at 10 µg/g (dry soil basis). All containers were then shaken vigorously. The mouth of each bottle was closed with a screw cap and the top of each flask was covered with aluminum foil. The headspace for each 1-L flask, 1-L media bottle, and 0.5-L media bottle was approximately 520, 607, and 250 cm³, respectively. These containers were incubated at 24 C in the dark. After a designated period (weekly, unless specified), the bottle was vigorously shaken and an aliquot of 1.8 ml soil-water mixture (approximately 2 g) was quickly drawn and placed into a 30 ml-bottle, which was covered with a screw cap with a Teflon-coated septum. These bottles were then stored in the freezer before the hexane

extraction as described in previously (Chapter 2). Each treatment was duplicated. This experiment was only conducted once due to prolonged incubation time.

4.2.4 Effect of antibiotics and fungicides on dechlorination of TB in a DPS-

conductive soil. To test which group of microorganisms might be implicated in DPS, selected antibiotics or fungicides were incorporated into a DPS-conductive soil to see if the dechlorination of TB was affected. Two grams of South Farm soil was mixed with 3 ml of sterile, distilled water in a test tube and incubated for 3 weeks in the dark. TB was added to a concentration of 20 µg/g. Each of the antibiotics kasugamycin hydrochloride, bacitracin, polymyxin B sulfate, ampicillin, tetracycline, streptomycin sulfate, vancomycin hydrochloride, and cyclohexamide was added at 100 µg/g, while each of the fungicides Ridomil 2 E, Mancocide, Kocide, and PCNB were added at 500 µg/ml of formulated products. The contents of the test tubes were mixed by vortexing. All test tubes were incubated in the dark for 2 weeks at room temperature (approximately 25 C). The contents of each tube was then vortexed and washed twice with 1 ml sterile, distilled water into a tube with a Teflon-coated cap. Then 20 ml of hexane was added and the tubes were transferred to a shaker at 300 rpm for 15 minutes. The hexane was then transferred into another tube and evaporated. Two ml of fresh hexane was added to each tube and mixed. The hexane was then subjected to GC/MS analysis to determine concentrations of TB and DTB. The experiment was conducted twice.

4.2.5 Comparison of microorganisms in soil suspensions with or without

dechlorinating activity. This experiment compared the aerobic microbial profiles of actively dechlorinating (active) and nonactively dechlorinating (non-active) suspensions which were prepared from the same conductive soil, and determine whether

these microbes were capable of dechlorinating TB after being inoculated back into an autoclaved soil suspension or soil extract. In previous test of acclimation of TB in different types of containers, the two 1L- media bottles initially treated with TB exhibited different dechlorinating activities: one was actively dechlorinating TB while the other was not dechlorinating TB up to 162 days after incubation. These two suspensions were chosen. Three types of media were selected: nutrient agar (NA), potato dextrose agar (PDA), and soil extract agar (SEA). The PDA, NA and agar were all purchased from Difco Laboratories, Detroit, MI. PDA and NA were prepared following manufacturer's directions. SEA was prepared by adding 15 g/L of agar into soil extract. One kg of South Farm soil was added into a 2-L flask, and then 1L of distilled water was added. The flask was covered with aluminum foil and autoclaved (15 psi 30 min). The contents were filtered through cheesecloth and then through a #4 filter paper. The filtrate was adjusted to a volume of 1 L with distilled water and the pH was adjusted to 7.0 with 1 N NaOH. Each medium was amended with TB at 0, 5 or 10 µg/ml (final concentrations).

Ten-fold dilutions of active and non-active suspensions were prepared in 9 ml of sterile 0.02 M phosphate buffer (pH 7.0) to a final dilution of 10^5 . An aliquot of 0.2 ml of each dilution was transferred to an empty, sterile Petri dish and 10 ml of medium was then added, swirled to mix and allowed to solidify. These plates were incubated at 28 C for 2 days and then kept at room temperature (23-25 C) for 2 days before colony types and colony numbers were counted.

To test if the microorganisms growing on the above media were capable of dechlorinating TB, microorganisms from selected plates were inoculated into autoclaved soil suspension and soil extract. The soil suspensions used for the inoculation were

prepared by mixing 10 g of South Farm soil with 20 ml of sterile, distilled water in 100 ml-flasks. The soil extract used for the inoculation was prepared as described above, and 30 ml of soil extract was transferred into a 100- ml flask. These flasks were covered with aluminum foil and autoclaved on each of 2 consecutive days. TB was amended at 10 $\mu\text{g/ml}$ (final concentration) before inoculation with a microorganism.

4.2.6 Effect of continuous aeration on dechlorination of TB. To test the effect of aeration on dechlorination rate of TB, two incubation conditions were compared: still conditions (23-25 C) in which the soil suspension could create an anaerobic zone through stratification, and constant shaking (200 rpm, 23-25 C) to maintain aerobic conditions. Thirty ml of distilled water or soil extract from South Farm soil were transferred into 125-ml flasks and autoclaved for 20 minutes at 135 C. Aluminum foil was used to cover the top of the still treatment flasks while cotton was used to cover the top of the shaking treatment. TB was added at 20 $\mu\text{g/g}$ final concentration, then each flask was inoculated with 30 ml of an activated South Farm soil suspension in which TB had been repeatedly added and quickly dechlorinated. One ml of sample was removed from each flask at 0, 3, and 6 days after inoculation and extracted with 5 ml of hexane and subjected to GC/MS analysis to determine TB and DTB concentrations. Each treatment had two replications. The experiment was conducted twice.

4.2.7 Effects of oxygen exclusion/replacement on the dechlorination of TB. To determine the effects of excluding oxygen or repeated application of TB on dechlorination of TB, serum bottles were first flushed with a syringe needle with N_2/CO_2 (80% N_2 plus 20% CO_2), covered with a butyl stopper, and then autoclaved. Sterile 125 ml- flasks covered with aluminum foil were used as the control. Thirty ml of undiluted

old suspension, enriched over a year through repeated application of TB into the suspension, or freshly enriched suspension, were injected into the serum bottles or transferred into flasks. For the first application, TB dissolved in ethanol was applied at either 10 or 50 µg/ml (final concentration). TB was re-applied to all treatments at 20 µg/ml 5 days after the first application. All treatments were incubated at room temperature (24-25 C) in the dark, and 1-ml samples were collected at 0, 2.5, 5, 6, and 7 days, extracted with 5-ml hexane and subject to GC/MS. Each treatment had two replications. The experiment was conducted twice.

4.2.8 Effects of fungal and bacterial inoculation and additional nutrients on the dechlorination of TB in greenhouse studies. South Farm soil (conductive soil) was placed into autoclavable bags at 1.8 kg/bag, and autoclaved for 1 h. The bags were stored at room temperature for 1 day and re-autoclaved for 1 h. Each bag of soil was transferred into a plastic pot; the top of the bag was opened and filled with tap water. Fungal isolates (one *Fusarium* sp. isolate and one *Trichoderma* sp. isolate) and an unidentified bacterial isolate (isolated previously from conductive soil) were inoculated either individually or in mixtures of different organisms. TB was applied at 4.5 kg ai/ha based on the soil surface area. Rice seedlings (cv. Bengal), pre-germinated in autoclaved greenhouse soil and grown in flats for 10-14 days were transplanted into each pot at two seedlings/pot. The water level in each pot was maintained with tap water. After 20-30 days, a soil sample was collected from the contents of the pot using a soil corer. Samples were stored in a freezer until extraction with hexane. Five grams of soil were mixed with 5 ml of hexane in a 30 ml-bottle, which was covered with a screw cap with a Teflon-coated septum. The mixture of hexane and soil in the bottle was incubated on an orbital shaken at 400 rpm for

30 min. Approximately 0.1 ml of hexane was transferred into an insert for the auto-sampler of GC/MS as described previously. The experiment was conducted twice.

4.2.9 Attempts to isolate anaerobic dechlorinating microorganisms. In order to see if there were any currently known reductive dehalogenators involved in the dechlorination of TB in South Farm soil, different anaerobic media, which were known to support reductive dechlorination, were tested. The procedures for the preparation of the media were modified from those described previously (Cole et al. 1994; DeWeerd et al. 1990; Sanford et al. 1996; Utkin et al. 1994). The media, which were used successfully to isolate facultative anaerobic bacterium “2CP-1” (Cole et al. 1994), strict anaerobic bacterium *Desulfomonile tiedjei* (DeWeerd et al. 1990), *Desulfitobacterium chlororespirans* (Sanford et al. 1996), *Desulfitobacterium dehalogenans* (Utkin et al. 1994) and *Dehalospirillum multivorans* (Scholz-Muramatsu et al. 1995), were tested for the ability to support dechlorination of TB. Briefly, heat-stable components of a medium were mixed in a 1 L-media bottle and heated in a microwave oven until boiling. The headspace of the bottle was continuously flushed with N₂/CO₂ (80:20) gases until the liquid in the bottle cooled to room temperature. Reducing agents were then added and mixed. Ten to 30 ml of each medium was then transferred into a 110 ml-serum bottle, which had been flushed with the same N₂/CO₂ mixture. The serum bottles were then covered with butyl stoppers and sealed with aluminum caps before autoclaving at 15 psi for 20 min. Before inoculation with suspension or organism, vitamin mixtures were injected through the butyl stopper using 1 ml-syringe (Beckton Dickinson Co.). An actively dechlorinating suspension (prepared with South Farm soil) was used as an inoculum at five different levels: 0.01, 0.1, 0.5, 1, 5 and 10%. The serum bottles were

then incubated at room temperature in the dark. Dechlorination of TB was monitored periodically (first weekly and then monthly).

4.2.10 Quantification of TB and DTB with GC/MS. GC/MS was run on a Hewlett Packard GC 6890 Gas Chromatograph equipped with a HP 7683 Injector and a HP5973 Mass Selective Detector. The injector temperature was held at 250 C. The initial oven temperature was 40 C for 3 min, the temperature was then ramped to 280C at 12C/min and held at 280 C for 5 min. Helium was used as the carrier at a flow rate of 23.6 ml/min. A 30-m-long by 0.32-mm-i.d. DB-5 capillary column (film thickness, 0.25 μ m) from J & W Scientific was used. Selected ion monitoring (SIM) mode was used, which selected multiple characteristic target ions m/z 72.1, 91.1, 100.1, and 223.1 for DTB; and m/z 72.1, 100.1, 125, and 257 for TB.

4.2.11 Statistical Analysis. Standard deviation, standard errors, and correlation coefficients were calculated using Excel 2000 (Microsoft Inc.). Multiple comparisons were performed using SAS 8.0 with Fisher's protected LSD.

4.3 Results

4.3.1 Effects of sterilization of DPS-conductive soil on dechlorination. In the greenhouse experiment, DPS only occurred in the treatment with nonautoclaved conductive soil treated with either the Bolero 8 EC or 10 G (Figure 4-1). DPS plants were stunted, averaging 28 to 46 cm in height (Table 4-1). The conductive soil treated with Bolero 10 G showed the shortest mean plant height at 28.0 cm, compared to a mean height of 69.8 cm in the same soil without Bolero 10 G. DPS symptoms first showed 2 weeks after transplanting. DPS did not occur in autoclaved soil, regardless of Bolero treatment. The treatments with non-conductive soil (North Farm) did not show DPS,

regardless of whether Bolero was applied or not (Table 4-1). All the treatments with autoclaved soil had taller plants. The treatments that showed DPS also had reduced yields and generally more tillers than other treatments, which did not show DPS. An exception was the autoclaved treatment with DPS-conductive soil without Bolero, which also had an increased number of tillers (Table 4-1).

4.3.2 Dechlorination acclimatization and enrichment.

In 1-L flasks initially treated with TB, dechlorination started after 14 days incubation (DAI) (Figure 4-2A). By 21 DAI, the percentage of DTB in the suspension increased from 0 to 23%. Dechlorination of TB was almost completed by 28 DAI, with 96% of the TB being converted to DTB. After all TB was depleted (transformed into DTB), TB was then added at 49 DAI. In the flasks initially treated with TB, DTB increased from 27 to 77% within 7 days (by 56 DAI). The remaining TB was dechlorinated within another 7 days (by 63 DAI).

For those initially treated with DTB, the dechlorination of TB did not start within the first 7 days, but all TB was dechlorinated after an additional 7 days (63 DAI). At 77 DAI, additional TB was added into each flask, and DTB and TB concentrations were monitored at 2-day intervals for 8 days. The dechlorination rates were very similar regardless of whether the suspensions were initially treated with TB or DTB. All TB was dechlorinated within 4 days (by 81 DAI). TB was again added into each flask at 162 DAI after a long period of no TB exposure (81 days). The dechlorination was slower than previous application of TB, with less than 50% DTB in both treatments within 4 days (by 166 DAI).

Table 4-1. Effect of autoclaving on development of delayed phytotoxicity syndrome (DPS) on rice in conducive and non-conductive soils

Soil type ^X	Autoclaving treatment	Bolero ^Y added	Rice height ^Z (cm)	Rice yield ^Z (g)	Rice tiller ^Z number
Nonconductive	Yes	0	86.3 a	6.4 ab	4.0 bcd
Nonconductive	No	10G	60.5 bc	2.2 cd	2.0 d
Nonconductive	No	0	70.8 ab	2.5 cd	2.0 d
DPS-conductive	No	8EC	46.5 cd	0.6 de	7.8 a
DPS-conductive	No	10G	28.0 d	0.2 e	7.0 a
DPS-conductive	No	0	69.8 ab	1.4 cde	3.5 cd
DPS-conductive	Yes	8EC	76.3 ab	5.4 b	6.8 ab
DPS-conductive	Yes	10G	85.5 a	7.5 a	5.5 abc
DPS-conductive	Yes	0	85.8 a	7.9 a	7.8 a

^X DPS-conductive soil was Crowley silt loam soil from the South Farm Unit, and DPS-nonconductive soil was from the North Farm Unit of the LSU Rice Research Station, Crowley, LA.

^Y The Bolero was applied at a rate of 4.5 kg ai/ha for those treated with either 8EC or 10G Bolero.

^Z Different letters within columns indicate statistical significance at p=0.05.



Figure 4-1. Greenhouse test of microbial involvement in delayed phytotoxicity syndrome (DPS). Autoclaved or nonautoclaved South Farm soil (DPS-conductive) were mixed with thiobencarb (Bolero 8 EC or 10 G) at the rate that was equivalent to 4.5 kg ai/A. One-week old rice seedlings were transplanted at two seedlings/pot. From left to right: 1. autoclaved soil (CK); 2. nonautoclaved soil (CK); 3. autoclaved soil treated with Bolero 8 EC; 4. nonautoclaved soil treated with Bolero 8 EC; 5. autoclaved soil treated with Bolero 10 G; and 6. nonautoclaved soil treated with Bolero 10G.

In 1-L media bottles (Figure 4-1B), the dechlorination was generally slower than in 1-L flasks. The dechlorination did not start within 21 DAI for those initially treated with TB, and only 36% of the TB was converted to DTB after 36 DAI. When TB was added at 49 and 77 DAI, dechlorination occurred rapidly in those suspensions initially treated with TB, while those initially treated with DTB showed slower conversion rates. When TB was added at 162 DAI, the dechlorination rates were similar for those initially treated with TB and those initially treated with DTB.

In 0.5-L media bottles, no dechlorination of TB occurred up to 210 DAI for the treatment initially treated with TB (Figure 4-1C). Those initially treated with DTB had a slight increase in DTB percentage after TB was added at 49 DAI. However, dechlorination of TB was still incomplete by 162 DAI, with 52% being converted to DTB. When TB was added at 209 DAI, the dechlorination rate was higher (Figure 4-2).

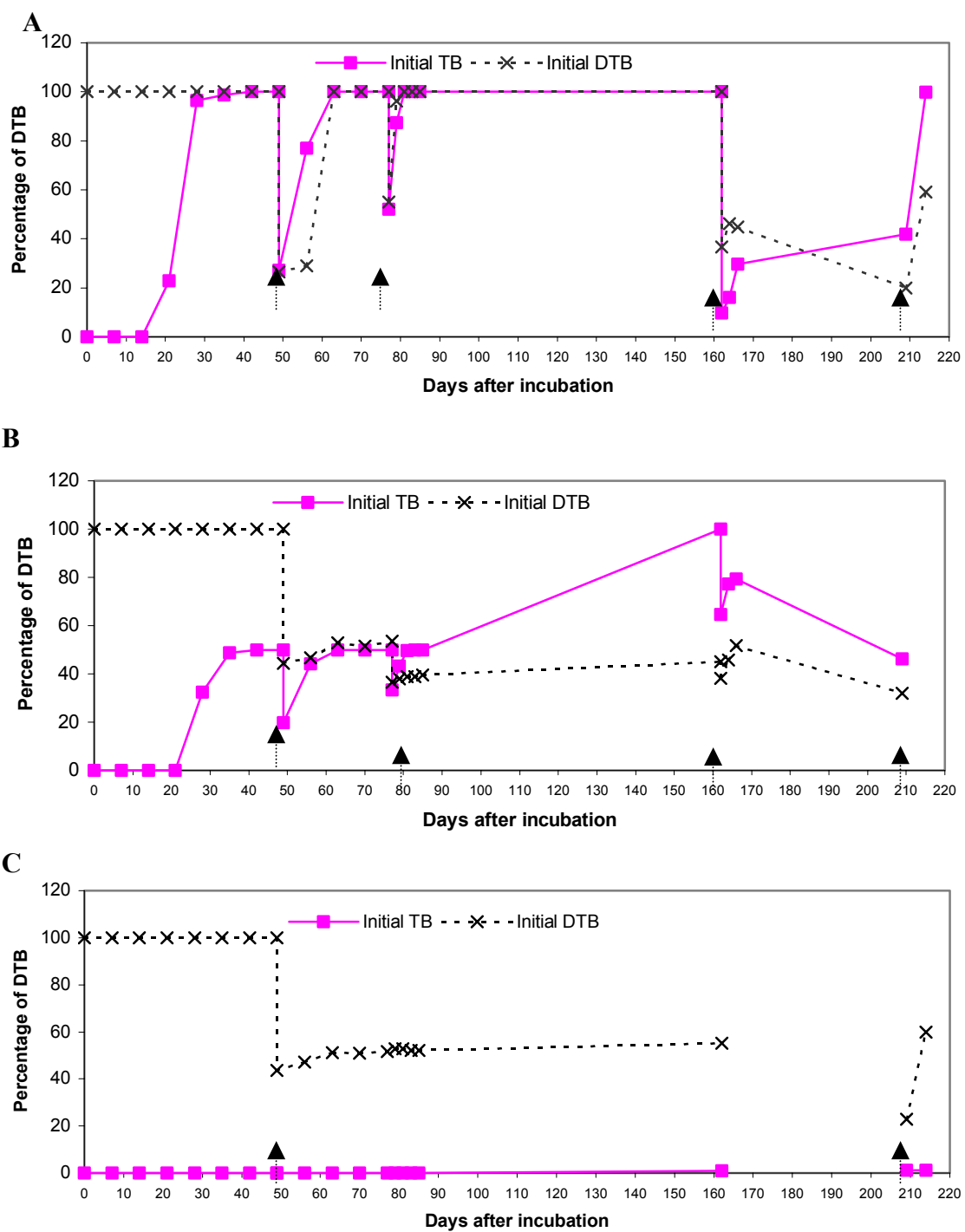


Figure 4-2. Effects of repeated application of thiobencarb (TB) on the dechlorination of TB in soil suspensions initially incubated with TB or DTB using 1-L Flasks (A), 1-L media bottles (B), or 0.5-L media bottles (C) as containers. Data are the average values of duplicated treatments. Soil suspensions were prepared from DPS-conductive soil. Arrows indicate the times that additional TB was added.

The pH of suspensions in each container was checked at the end of the experiment. Suspensions in 1-L flasks, 1-L media bottles, and 0.5-L media bottles, had pH values of 7.0-7.5, 6.2-6.7, and 5.0-5.5, respectively.

4.3.3 Effect of antibiotics and fungicides on dechlorination of TB in a DPS-

conductive soil. In the control with only TB added, 39% of TB was converted into DTB. At 100 µg/ml, the antibiotics kasugamycin, bacitracin, polymyxin, ampicillin, penicillin, tetracycline, streptomycin, vancomycin, and cycloheximide completely inhibited the dechlorination of TB to DTB in South Farm soil during the testing period (2 weeks). At 500 µg/g, the fungicides Mancocide and Kocide inhibited dechlorination. The treatment with PCNB had no inhibitory effect on dechlorination of TB; with 35% TB converted into DTB. Treatment with Ridomil reduced dechlorination to 1.5%.

4.3.4 Comparison of microorganisms in soil suspensions with or without

dechlorinating activity. The non-dechlorinating suspension had significantly more bacteria and fungi than the active suspension, regardless of the media used and the concentrations of TB (Table 4-2). The differences among the three media were not significant, except that nutrient agar had no fungi from the active suspension, regardless of TB concentration.

When microorganisms growing in different types of plates were mass inoculated into a soil extract or soil suspension, organisms from SEA, NA, and PDA plates did not dechlorinate TB up to 5 weeks after incubation, while the controls, which were inoculated with an activated suspension (Table 4-3), showed dechlorination in both soil extract and the soil suspension.

Table 4-2. Estimation of bacterial and fungal colony numbers from activated and nonactivated thiobencarb (TB) dechlorinating suspensions in different media

Medium ^X	TB (µg/g)	Bacterial colony (x10 ⁵) ^Y		Fungal colony (x10 ³) ^Y	
		Active ^Z	Non-active ^Z	Active	Non-active
SEA	0	13.0 (4.4)	59.6 (8.4)	0.3 (0.6)	2.67 (1.2)
	5	9.0 (3.4)	58.0 (1.0)	0	1.00 (0)
	10	9.6 (2.3)	47.3 (7.0)	0	3.33 (1.5)
PDA	0	16.6 (4.7)	55.6 (14.6)	1.33 (1.5)	1.67 (1.5)
	5	17.6 (4.0)	51.3 (14.0)	1.67 (1.1)	1.00 (1.0)
	10	15.6 (5.7)	64.3 (0.6)	0	2.33 (1.2)
NA	0	8.3 (4.0)	56.0 (3.6)	0	1.33 (1.5)
	5	12.0 (6.2)	51.6 (3.2)	0	0.33 (0.6)
	10	10.0 (2.6)	N/A	0	2.67 (2.1)

^XSEA, soil extract agar; PDA, potato dextrose agar; NA, nutrient agar.

^YData are the colony forming units of bacteria and fungi (standard deviation) per ml of soil suspensions.

^ZOne active dechlorinating suspension and one non-dechlorinating suspension were compared.

Table 4-3. Conversion of thiobencarb (TB) to dechlorinated thiobencarb (DTB) by microbes growing in different media plated with dilutions of activated suspensions.

Type of plates used to inoculate ^W	Soil/soil extract ^X	DTB detected ^Y
SEA (5 µg/ml TB)	SE	-
SEA (10 µg/ml TB)	SE	-
SEA (0 µg/ml TB)	SE	-
NA (5 µg/ml TB)	SE	-
PDA(10 µg/ml TB)	SE	-
NA 10 µg/ml TB	SE	-
Activated suspension (CK)	SE	+
SEA (5 µg/ml TB)	Soil	-
SEA (10 µg/ml TB)	Soil	-
SEA (0 µg/ml TB)	Soil	-
NA (5 µg/ml TB)	Soil	-
PDA (10 µg/ml TB)	Soil	-
NA (10 µg/ml TB)	Soil	-
Activated Suspension (CK)	Soil	+

^W Three different media with different concentrations of TB added into the media. SEA, soil extract agar; PDA, potato dextrose agar; NA, nutrient agar.

^X SE: sterile soil extract from conducive South Farm soil. Soil: autoclaved conducive South Farm Soil.

^Y DTB was monitored from week 1 to week 5 after inoculation using GC/MS. +: DTB detected; -: No DTB detected.

4.3.5 Effect of aeration on dechlorination of TB. Flasks incubated under high-speeded shaking (200 rpm), and thus well aerated, maintained a redox potential at more positive values (approximately Eh +300 mV) than flasks incubated under still conditions. In still flasks, the Eh reached –200 mV. A comparison of TB and DTB concentrations at day 0, 3 and 7, is shown in Figure 4-3.

When the soil extract was used to dilute the activated soil suspension prior to incubation under still conditions, dechlorination of TB occurred. TB concentration in the suspension decreased while DTB concentrations increased (Figure 4-3A). Under conditions of continuous aeration, no dechlorination occurred, both TB and DTB remained constant for 3 days. The residual amount of DTB, which was carried over from the initial inocula, decreased and eventually disappeared after 7 days. The concentrations of DTB from conversion of TB, however, remained relatively unchanged up to 7 days.

When sterile, distilled water was used to dilute the activated soil suspension and incubated under still conditions, dechlorination occurred. TB was dechlorinated rapidly as indicated by the increasing DTB concentrations and concurrent decrease of TB concentrations (Figure 4-3B). Under shaking conditions, concentrations of both TB and DTB remained relatively constant during the incubation period.

The flasks that were incubated under shaking conditions were removed from the shaker after 7 days incubation and incubated again under still conditions. Dechlorination of TB was not detected after 7 additional days of incubation.

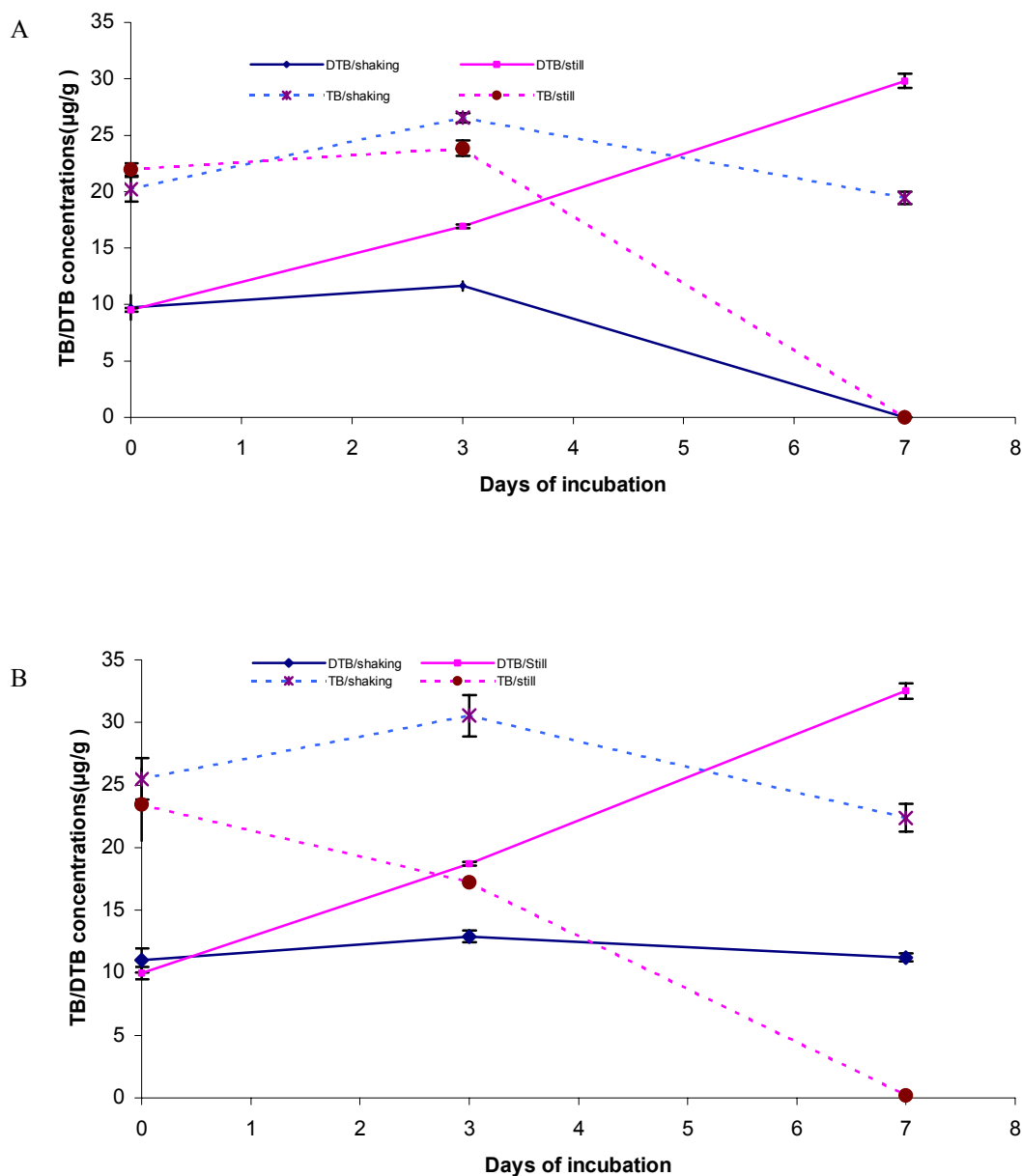


Figure 4-3. Effect of continuous aeration on dechlorination of thiobencarb (TB) in activated soil suspensions diluted with water or soil extract. The continuous aeration was represented by shaking at 200 rpm to generate aerobic conditions; still incubation in the dark was used as the control. A. The activated soil suspension diluted (1:1) with sterile soil extract. B. The activated soil suspension diluted (1:1) with sterile, distilled water. DTB: dechlorinated thiobencarb. Error bars represent standard deviations.

4.3.6 Effect of reducing agents on dechlorination of TB. Three different media were used: mineral medium, soil extract, and the equal combination of mineral salt medium and soil extract (Table 4-4). Dechlorination of TB only occurred in controls that did not have reducing agents, regardless of media used.

Table 4-4. Effect of reducing agents on reductive dechlorination of TB.

Reducing agent ^X	Medium ^Y	DTB detected ^Z
CK	M	+
0.025% Cysteine-HCl	M	-
0.02% Dithiothretol	M	-
CK	M + SE	+
0.025% Cysteine-HCl	M + SE	-
0.02% Dithiothretol	M + SE	-
CK	SE	+
0.025% Cysteine-HCl	SE	-
0.02% Dithiothretol	SE	-

^XEach reducing agent was prepared in a stock solution and added into the medium at a final concentration (%) as indicated. CK: no reducing agent was added.

^YMedia used in this test include: M, mineral salts medium modified from Moon and Kuwatsuka 1985a; SE, soil extract prepared from DPS-conductive soil, M+SE, a mixtures of M and SE in equal proportion.

^ZDTB was determined 14 days after inoculation by GC/MS analysis. +: DTB detected; -: No DTB detected

4.3.7 Effect of TB concentration and oxygen exclusion on the reductive

dechlorination of TB. Flasks covered with aluminum foil allowed the interchange of air between the headspace and the outside of the flasks. In serum bottles, the headspaces were filled with N₂/CO₂ and their mouths were sealed with butyl stoppers to prevent air exchange. The availability of oxygen to soil suspensions greatly affected dechlorination rates. In flasks amended with 10 µg/g of initial TB, the TB was completely dechlorinated 3 days after being added, regardless of whether the old suspension or the newly enriched suspension was used to inoculate flasks (Figure 4-4) or serum bottles (Figure 4-5).

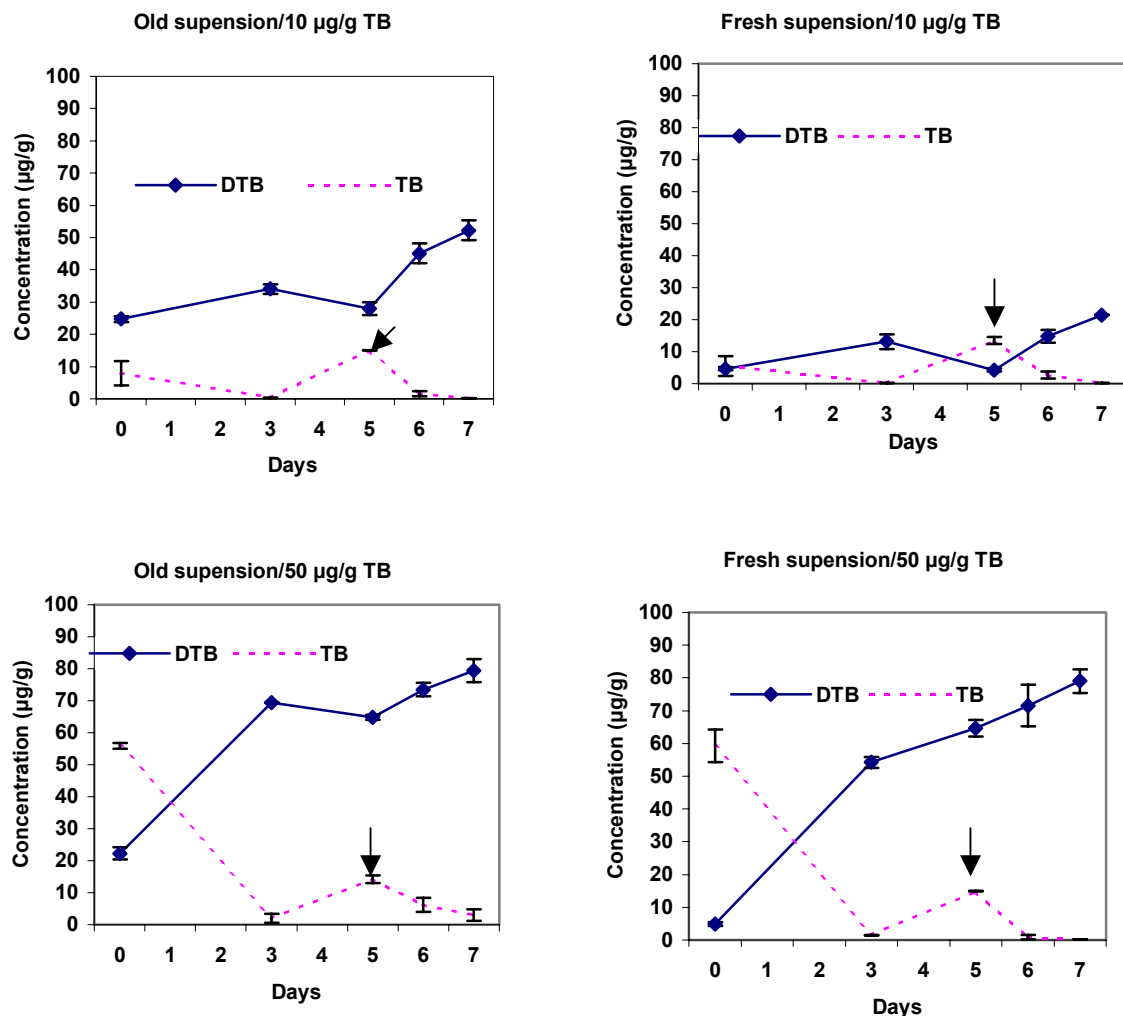


Figure 4-4. Results from flask containers in an experiment to determine the effects of oxygen exclusion and thiobencarb (TB) concentrations on dechlorination of TB. Thirty ml of either undiluted old suspension, which had been enriched over a year through applying TB periodically, or freshly enriched suspension, which had its first TB application 1 week earlier, were transferred into the flasks. For the first application, TB dissolved in ethanol was applied at either 10 or 50 $\mu\text{g/g}$ (final concentration). TB was re-applied to all treatments at 20 $\mu\text{g/g}$ (arrow) 5 days after the first application. Error bars represent standard errors.

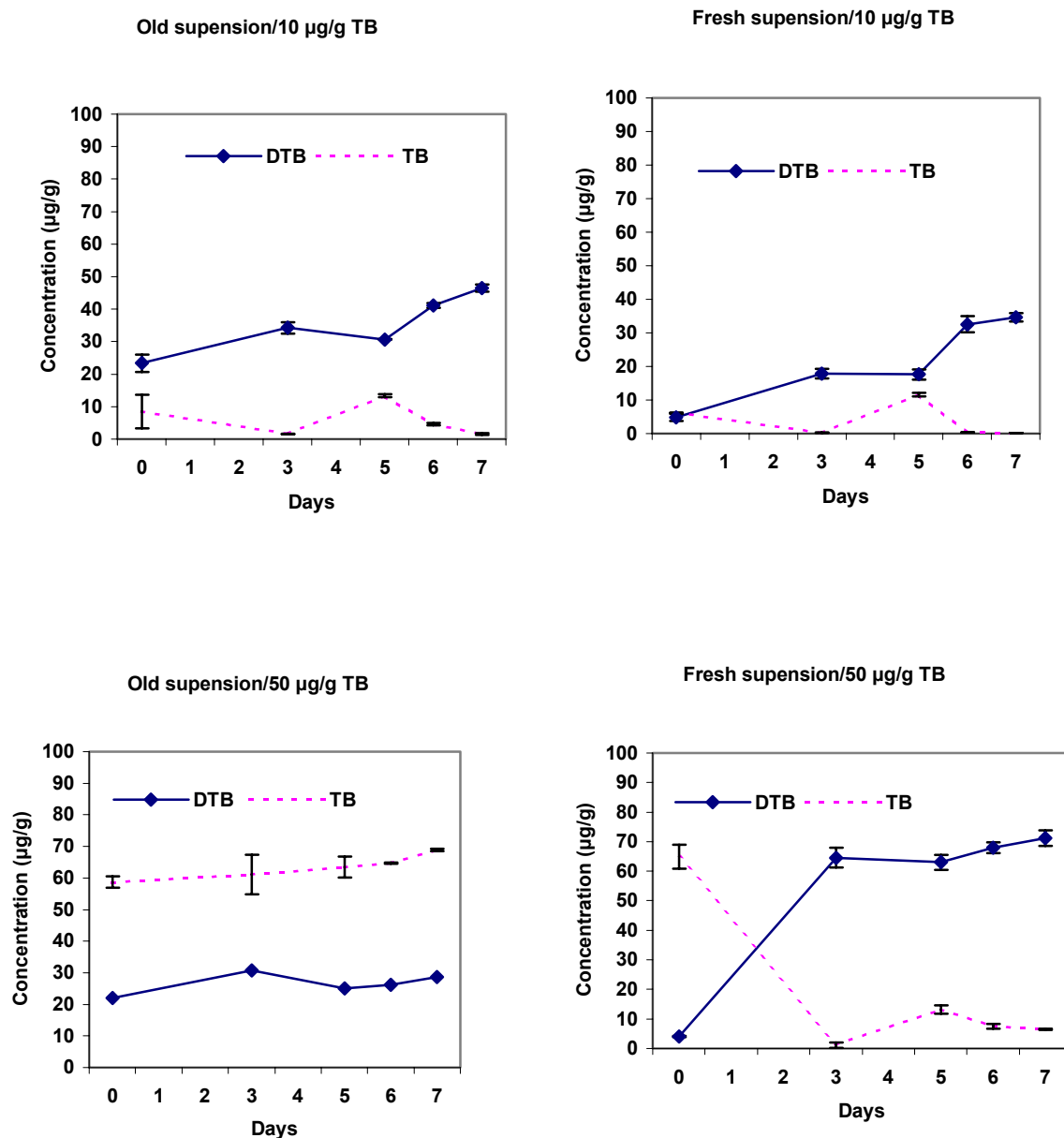


Figure 4-5. Results from serum bottle containers in an experiment to determine the effects of oxygen exclusion and thiobencarb (TB) concentrations on dechlorination of TB. Thirty ml of either undiluted old suspension, which had been enriched over a year through applying TB periodically, or freshly enriched suspension, which had its first TB application 1 week before the test, were transferred into the serum bottles, which had been flushed with N_2/CO_2 . For the first application, TB dissolved in ethanol was applied at either 10 or 50 µg/g (final concentration). TB was re-applied to all treatments at 20 µg/g (arrow) 5 days after the first application. Error bars represent standard errors.

When TB was applied at 50 µg/g, all TB added was dechlorinated after 3 days of incubation in flasks. In serum bottles, TB was dechlorinated within 3 days in freshly enriched suspension. TB was not dechlorinated in the old suspension when TB was amended at 50 µg/g. The TB concentrations remained relatively unchanged during the 7 days observation period.

When TB was added a second time, it was again quickly and completely dechlorinated in the treatments that had low TB concentrations after the first application of 10 µg/g TB. Those treated with higher TB concentrations (50 µg/g) in the first application, however, had slower rates of dechlorination, especially those inoculated with old suspension, or those using serum bottles as containers.

4.3.8 Effects of fungal and bacterial inoculation and additional nutrients on the dechlorination of TB in greenhouse studies. In the first experiment, 30 days after inoculation, there was a significant amount of DTB in the treatments with a *Fusarium* sp. or *Trichoderma* sp. or a bacterium added (Table 4-5). After 30 days, TB was mostly dechlorinated into DTB. There was slightly less DTB in the treatment of soil with TB with a bacterial isolate since it had a relatively higher amount of TB remaining. The non-inoculated control had little dechlorination. Inoculation with all three organisms showed lower amounts of TB and DTB than when they were inoculated individually. The TB-free controls showed no TB or DTB, regardless of whether inoculated or not. After incubating for 20 more days, the majority of TB in the soil was dechlorinated. However, sterile soil controls treated with TB also showed a high amount of DTB, indicating contamination with dechlorinating organisms.

Table 4-5. Dechlorination of thiobencarb in a conducive soil as affected by three different groups of added microorganisms under greenhouse conditions – the first experiment.

Inoculum	TB added	30 Day ^Z		50 Days ^Z	
		TB	DTB	TB	DTB
Ck	No	0 c	0 d	0 b	0.02 d
<i>Fusarium spp.</i>	No	0 c	0 d	0 b	0.01 d
<i>Trichoderma spp.</i>	No	0 c	0 d	0 b	0.03 d
Unidentified bacterium	No	0 c	0 d	0 b	0.02 d
Mixture of 3	No	0 cd	0.01 d	0 b	0.02 d
Ck	Yes	1.78 a	0.08 d	0.44 a	1.96 a
<i>Fusarium spp.</i>	Yes	0.35 c	2.19 b	0.03 b	0.89 bc
<i>Trichoderma spp.</i>	Yes	0.36 c	2.84 a	0.03 b	2.02 a
Unidentified bacterium	Yes	0.85 b	0.99 b	0.17 b	1.17 b
Mixture of 3	Yes	0.16 c	0.16 c	0.02 b	0.25 cd

^ZData are concentrations of thiobencarb (TB) or dechlorinated thiobencarb (DTB) in µg/g. Different letters within columns indicate statistical significance at p= 0.05.

In the second experiment (Table 4-6), two treatments were added to see if the nutrient alone would affect dechlorination. When sampled 30 DAT, most of the TB in the soil was dechlorinated, except the treatment of TB without any organisms, which had a slower rate of dechlorination (0.23 µg/g) and was left with a high concentration of TB (1.78 µg/g). The treatment with TB with additional nutrients, however, showed dechlorination at a rate comparable to those inoculated with individual or mixture of organisms. In the second sampling at 50 DAT, there was slightly more DTB in the treatment of TB without additional nutrient.

Table 4-6. Dechlorination of thiobencarb in a conducive soil as affected by three different groups of added microorganisms under greenhouse conditions-the second test.

Inoculum	TB added	30 Day ^Z		50 Days ^Z	
		TB	DTB	TB	DTB
Ck	No	0 b	0 c	0 b	0 d
<i>Fusarium</i> spp.	No	0 b	0 c	0 b	0 d
<i>Trichoderma</i> spp.	No	0 b	0 c	0 b	0 d
Unidentified bacterium	No	0 b	0 c	0 b	0 d
Mixture of 3	No	0 b	0 c	0 b	0 d
Nutrient broth	No	0 b	0 c	0 b	0 d
Ck	Yes	1.04 a	0.23 bc	0.19 a	0.86 bc
<i>Fusarium</i> spp.	Yes	0.03 b	0.68 a	0.02 b	1.59 a
<i>Trichoderma</i> spp.	Yes	0.03 b	0.68 a	0.01 b	0.50 cd
Unidentified bacterium	Yes	0.05 b	0.32 bc	0.02 b	1.38 ab
Mixture of 3	Yes	0.02 b	0.45 ab	0.02 b	0.85 bc
Nutrient broth	Yes	0.04 b	0.43 ab	0.02 b	1.04 abc

^ZData are concentrations of thiobencarb (TB) or dechlorinated thiobencarb (DTB) in µg/g. The letters in each column indicate statistical significance at the level of 0.05.

4.3.9 Attempts to isolate dechlorinating organisms. There were anaerobic or facultative anaerobic organisms growing in the anaerobic media inside the serum bottles. The media became turbid as the incubation continued. The liquid media turned black and liquid withdrawn from the media had a strong smell similar to H₂S. However,

dechlorination of TB was not detected with GC/MS in all the anaerobic media, even after an extended incubation time (up to 6 months). A series of dilutions of the inocula also failed to support the dechlorination. The pH remained constant during the incubation period in the range of 7.0- 7.5. Repeated attempts to enrich or to isolate dechlorinating organisms from these media were not successful.

4.4 Discussion

The role of microorganisms in dechlorination of TB was studied. Sterilization of DPS-conductive soil prevented the development of DPS after addition of TB, suggesting that DPS in south Louisiana rice fields was biologically dependent. Using suspensions prepared from conductive soil, it was also shown that dechlorination of TB in soil suspensions required the initial presence of oxygen. However, excessive oxygen provided by shaking at high speed (200 rpm) inhibited dechlorination of TB. Although dechlorination only occurred in reduced zones in a soil column, adding reducing agents (cysteine and dithiothreitol) inhibited dechlorination in soil suspensions. It appeared that many microorganisms might affect dechlorination of TB in soil suspensions, as dechlorination was inhibited by several antibiotics, and was slowed or inhibited by three fungicides. Two of the fungicides were copper compounds and would be expected to have bactericidal activity.

There appeared to be fewer populations of aerobic microorganisms in soil suspensions that exhibited dechlorinating activity. These microorganisms were not able to dechlorinate TB when inoculated into sterile soil suspensions in flasks. However, two fungi selected from this group of organisms were able to enhance the dechlorination of

TB in pot-soil in the greenhouse. The additional nutrient that came with the inocula might have also contributed to the enhanced dechlorination rates.

In the test where conducive soil was sterilized, TB and DTB concentrations were not quantified from soil or plant samples. The presence of rice plants showing DPS-like symptoms, such as dark green color, stunting, twisting of leaves and stems, and excessive tillering, were used as indications of dechlorination of TB in the soil. Thus, results from this test should be considered preliminary. Nevertheless, the treatments of conducive soil with TB significantly reduced plant heights, reduced grain yield and enhanced tiller numbers compared to the no TB treatments or to sterilizing treatments. The treatments of rice field soil collected from the North Farm of the LSU Rice Research Station (considered nonconductive) with different concentrations of TB, did not cause significant reductions in plant height, grain yield, or tiller numbers as compared to the no TB control. This soil was considered nonconductive as previous application of TB to field plots did not result in DPS-like plants.

In previously published studies, the lag period for subsequent dechlorination was reduced from that of earlier applications and the populations of dechlorinating microbes appeared to increase after TB application, resulting in the enrichment of dechlorinating activity (Moon & Kuwatsuka 1984, 1985a, 1985b, 1985c, 1987b). However, a critical control, in which the suspension was incubated under the same conditions, but in which TB was not added until the second or the third application, was not included. This control might have made it possible to determine if shortening of the lag period of subsequent applications resulted from a more suitable dechlorinating environment rather than by enhancement due to previous applications of TB.

In the dechlorination acclimation and enrichment test, the lag period before dechlorination of TB was shortened by the previous application of TB, but not DTB, into flooded rice field soil. This suggested that dechlorinating microbes were enriched through repeated application of TB (Moon & Kuwatsuka 1984, 1985a). Theoretically, the reductive dechlorination of TB yields 130 to 160 KJ/mol of energy with hydrogen as an electron donor under standard conditions (pH 7, 25 C, 1 molar concentration of TB, 1 atm. hydrogen partial pressure) (J. Dolfing, personal comm.). This amount of energy is sufficient to support the growth of microorganisms. However, enrichment by the repeated application of TB was limited. Enrichment, as indicated by the shortening of the lag period (through the enrichment of dechlorinating organisms) was only significant in the 1L-flasks in the second (49 DAI) and third (77 DAI) application of TB (Figure 4-2). By the fourth or fifth application, the dechlorination rates were reduced. One explanation would be that the substances required for dechlorination were being exhausted (such as those that serve as electron donors) or toxic substances accumulated.

The experimental system itself affected dechlorination. The 1-L flask appeared to be better for dechlorination of TB than media bottles. The flasks may allow more air exchange, as they were loosely covered with aluminum foil rather than tightly covered with a screw cap as with the media bottles. Furthermore, the headspace of the 1-L flask and 1-L media bottle were twice as large as with the 0.5-L media bottle. The air in the headspace may help aerobic organisms to grow and create an environment that is more amenable for the reductive dechlorination of TB. This also can be confirmed by the observation that dechlorinating activities in 1 L-media bottles were greater than those in the 0.5 L media bottle. A check of pH of the suspension inside the flasks or media bottles

revealed that suspensions inside flasks had neutral or close to neutral pH, which favored the dechlorination of TB in the suspension (Moon and Kuwatsusa 1985a), while those inside media bottles were acidic (pH 5.0-5.5 in 0.5-ml media bottles).

In the test of the effects of antibiotics and fungicides on dechlorination of TB, many antibiotics inhibited dechlorination, including those that inhibit eukaryotes (cycloheximide) and prokaryotes (such as ampicillin, penicillin, and streptomycin). This test only examined dechlorination through 14 days incubation. Whether the dechlorination of TB would occur after longer incubation periods has not been tested. The effects of these antibiotics or fungicides on dechlorination of TB might be direct or indirect. The indirect effect would include effects on microorganisms that affect dechlorination by creating a favorable soil environment for dechlorination.

Populations of aerobic microorganisms in soil suspensions appeared to be affected by dechlorination of TB. Soil suspensions that were actively dechlorinating TB appeared to have fewer aerobic bacteria or fungi than those that were not actively dechlorinating. Whether this reduction in aerobic microorganisms resulted from a less favorable environment or from toxicity of DTB, which was transformed from TB in the suspension, was not clear. Those aerobes did not appear to be the dechlorinating microorganisms as they were not able to dechlorinate TB when inoculated into sterile suspension, either in mixtures or individually (data not shown).

Previous studies suggested that dechlorination of TB could only be detected under anaerobic or reductive conditions (Moon & Kuwatsuka 1985b). Redox potential measurements in a soil column also supported this observation (Chapter 3). This was further evaluated in the test using a high level of aeration, where the effects on

dechlorination of TB were measured. Under still conditions, the soil suspension would become stratified with aerobic and anaerobic zones from the water phase, water/soil interface and soil phase (Reddy et al. 1987). Studies indicated that the soil/water interface separates the aerobic and anaerobic zone of the soil column (Patrick et al. 1985). Starch was added to expedite development of anaerobiosis by stimulating the activity of microorganisms, thus enhancing consumption of oxygen in the suspension and also producing reducing substances. However, the addition of the starch or reducing agents may exert other effects, such as promoting the growth of microorganisms inhibitory to the dechlorinating organisms, or may make environmental conditions unfavorable for dechlorinating organisms or dechlorination. Indeed, dechlorination was not enhanced in our tests. The exclusion of oxygen may have similar effects. On the other hand, rigorous aeration (by shaking at 200 rpm) inhibited the dechlorination of TB, regardless of whether soil extract or distilled water was used to prepare the suspension. According to Stark (1996), shaking speeds of 180 rpm were necessary to ensure adequate aeration in soil slurries. This study showed that the dechlorination of TB is a complex problem.

An interesting question was raised in the study of continuous aeration on the dechlorination of TB. In the treatments in which soil extract was used to dilute the activated suspension at a 1:1 ratio, dechlorination of TB occurred when still conditions were used. However, under continuous aeration, no dechlorination of TB was observed. The residual DTB, which was carried over from the inocula, was completely degraded in this treatment. The TB remained unchanged during the same period. When distilled water was used instead of the soil extract, no degradation of the residual TB was observed during the incubation period. Substances inside the soil extract apparently

enhanced the growth of DTB degrading microorganisms. The mechanism of this degradation, such as whether it is a co-metabolic or energy-yielding process, remains to be resolved.

In the test to compare the effects of TB concentration and oxygen exclusion on the dechlorination of TB, an unexpected observation was that dechlorinating activity was not enriched by increasing concentrations of TB from 10 to 50 µg/ml or by repeated application of TB. In this test, TB appeared to be more readily dechlorinated in suspensions in which less TB was applied in prior applications. The dechlorination patterns differed from those described in aryl dehalogenation where energy from the reductive dechlorination was harvested by the dechlorinating organisms. A common observation is that a subsequent application dramatically reduced the lag period from the previous applications (DeWeerd et al. 1990; Löffler et al. 1997; Mackiewicz & Wiegel 1998; Sanford et al. 1996; Utkin et al. 1994).

In the greenhouse study conducted at Crowley, LA, dechlorination of TB occurred in all treatments where TB was applied, regardless of whether additional microorganisms were inoculated into the soil or not. Treatments amended with microorganisms, or nutrients, generally favored dechlorination. The dechlorination rate varied from treatment to treatment, or even between replications within the same treatment. As the soils used were autoclaved at least twice, it is likely that most organisms were eliminated. Tap water in the greenhouse could be a source for dechlorinating organisms, as well as, splashing of water from pot to pot.

Dechlorinating microorganism(s) were not isolated in this study. A variety of anaerobic media, which were supportive for the dechlorination of other halogenated

aromatic compounds, were tested for possible enrichment of dechlorinating microorganisms. All the media were inhibitory to dechlorination of TB. Soil suspensions lost their dechlorinating ability when transferred onto these media. In the experiment where the effect of adding reducing agents on dechlorination was tested, cysteine and dithiothreitol were inhibitory to the dechlorination of TB. The reducing agents, used in the preparation of other anaerobic media (Cole et al. 1994; DeWeerd et al. 1990; Sandford et al. 1996; Utkin et al. 1994) were toxic to dechlorinating microorganisms. One alternative choice would be hydrogen gas, which is an electron donor in many reductive dehalogenation reactions and is also a strong reducing agent (DeWeerd et al. 1991). Tsuchiya & Yamaha (1983; 1984) showed that the reductive dechlorination of 1,2,4-trichlorobenzenes by the intestinal contents of rats, or certain facultative anaerobic bacteria, required an atmosphere of 100% H₂. When the anaerobic media were incubated with in our laboratory with 100% H₂, instead of N₂/CO₂, the media were quickly reduced, but the dechlorination of TB did not occur even after incubation for 2 months (data not shown).

Flasks covered with aluminum foil were probably the best system to test for TB-dechlorinating microorganisms. Different sources of organisms were inoculated to test their dechlorinating capability. Many attempts to inoculate isolated microorganism individually or in mixtures failed to support dechlorination of TB in this system (data not shown). One explanation could be that the physical and chemical conditions in the refined flask system were not as favorable for the dechlorination of TB as that in pots in a greenhouse. Another explanation could be that the dechlorinating organisms were not

culturable in the media used. Thus, the identity of the dechlorinating organisms remains elusive.

Chapter 5

Summary and Future Research Needed

5.1 Summary

The delayed phytotoxicity syndrome (DPS) disorder in rice fields has been described for more than 20 years. It still remains a mystery in many aspects. Although the chemical basis for this disorder is the transformation of thiobencarb (TB) into dechlorinated thiobencarb (DTB), the phytotoxicity of TB, DTB, and the mixtures of TB and DTB to rice plants had not been quantitatively determined. The microbial nature of DPS had been suggested but no specific microorganisms determined. The overall objective of this dissertation study was to provide information to help characterize DPS problems in South Louisiana rice fields as to the following aspects: 1) to determine the phytotoxicity of TB, DTB and their mixtures under *in vitro* and greenhouse conditions; 2) to study the possible differentiation of sensitivity of different rice cultivars to TB and DTB; 3) to determine TB and DTB concentrations in rice plants showing DPS symptoms; 4) to address the mechanism of enhanced phytotoxicity of DTB by exploring the absorption and accumulation of TB and DTB in different parts of the rice plants; 5) to identify soil conditions affecting dechlorination; 6) to study the microbial factors affecting the transformation of TB into DTB; 7) and to determine where in soil columns microorganisms dechlorinated TB.

In the *in vitro* bioassay, which was developed in this study, rice seedling heights were reduced as concentrations of TB and DTB increased. In an *in vitro* test conducted in Magenta boxes, Bengal rice plants were slightly taller under low concentrations of TB (less or equal to 1 µg/ml) or DTB (less or equal to 0.1 µg/ml). However, rice plant

heights were inhibited as DTB increased to 1 µg/ml or TB increased to 10 µg/ml. In a more extensive *in vitro* study using test tubes, the phytotoxicity of TB, DTB, and mixtures of TB and DTB were evaluated using Lafitte rice. The effective dosages for 50% reduction in height were estimated at 6.2 µg/ml for TB and 0.3 µg/ml for DTB, indicating that DTB was highly toxic to rice. The injury to rice plants was enhanced when TB and DTB were combined at higher levels, suggesting that the toxic effects of TB and DTB to rice seedlings were additive.

A GC/MS method was developed to simultaneously detect TB and DTB concentrations in water, soil, and plant tissue samples. This detection method was consistent and accurate. In exploring the mechanism of toxicity of DTB to rice, it was determined that rice plants did not show preference in absorption and accumulation of DTB. Rice plants actually absorbed and accumulated more TB than DTB when TB and DTB were exposed to rice at equal concentrations. Rice cultivars exhibited differentiation in sensitivity toward TB and DTB. Among the four rice cultivars evaluated, M201 was more tolerant to TB and DTB than Bengal, Cocodrie and Lafitte.

The conditions affecting the transformation of TB into DTB in soil were studied through a special apparatus, which was developed to measure the redox potential of soil columns at different depths. The redox measurement apparatus showed consistency in the measurement of redox potential in a soil column. The relationship between reductive dechlorination of TB and the different redox conditions was analyzed by combining results of the redox measurements and results from the detection of TB and DTB concentrations. In the soil column, the dechlorination of TB peaked at the position that corresponded to an Eh of approximately – 230 mV 14 days after incubation.

Microbial aspects of reductive dechlorination of thiobencarb were studied. The dechlorination of TB in a soil suspension, which was prepared from a DPS-conductive soil, was affected by many factors. The excessive aeration caused by rapidly shaking (200 rpm) of the suspension inhibited the dechlorination of TB. Adequate air appeared to be necessary for the dechlorination of TB in a soil suspension, as the dechlorination was faster when the suspension was incubated in a 1-L flask, which was loosely covered with aluminum foil, than those incubated in 1-L and 0.5-L media bottles, which were covered tightly with a screw cap. The slow dechlorination rates in media bottles may have resulted from the effect of pH. The pH values of suspensions in media bottles were more acidic as compared to those in the flask system, which were close to neutral pH. This pH may have provided a more favorable environment for dechlorinating organisms. The exclusion of air from the suspensions reduced dechlorination more in old suspensions than in freshly prepared suspensions, especially when higher concentrations of TB were applied. Inoculation of one bacterial and two fungal isolates into DPS-conductive soils in the greenhouse resulted in higher rates of dechlorination. However, the same isolates inoculated into more defined sterile soil suspensions, prepared from DPS-conductive soil, failed to dechlorinate TB. Repeated attempts to isolate the organisms responsible for dechlorination of TB in Louisiana rice field soils were not successful.

5.2 Future Research Needed

5.2.1 Phytotoxicity

Research contributing to this dissertation demonstrated that TB and DTB were additive in causing injury to rice seedlings when rice seedlings were growing under sterile, *in vitro* conditions. While the *in vitro* assays have advantages, such as not having

the confounding effects of microorganisms and the complexity of the soil environment, they also share some disadvantages, e.g. results from *in vitro* studies may not truly reflect those from the fields. Greenhouse bioassays or even field tests should be conducted to provide additional evidence.

This study focused on four rice cultivars in testing the tolerance of rice cultivars to TB and DTB. Further tests should be tested with more rice cultivars or breeding lines to screen for cultivars/lines that are highly tolerant to TB and DTB.

5.2.2 Isolating dechlorinating microorganisms

Dechlorinating microorganisms have yet to be isolated and confirmed in a sterile and defined system. Results from this study indicated that the dechlorination of TB into DTB required first aerobic or near anaerobic conditions followed by reduced conditions e.g. redox potential around -200 mV. The microorganism(s) involved in dechlorinating TB probably require near anaerobic conditions for growth and dechlorination. Anaerobic conditions probably prevent the DTB from being decomposed by aerobic microorganisms, thereby allowing it to accumulate. The soil column developed in this study allowed these conditions to be developed *in vitro* and allowed the author to monitor the oxygen levels or Eh values. This system will be useful for isolating dechlorinating microorganisms from field soil and for their ability to transform TB to DTB *in vitro*. A common practice is to add reducing agents to achieve the reduced conditions. However, several reducing agents appeared to be toxic to the dechlorinating microorganisms or to aerobic or facultative anaerobic microorganisms involved in the dechlorinating system. Future study should explore identifying a reducing agent that is capable of reducing the

redox potential to the expected value (-200 mV) while not inhibiting the dechlorination of TB. A buffer system that is capable of maintaining pH around 7.0 is also critical.

References

- Ahrens, W.H., Anderson, C.D., Campbell, J.M., Clay, S., DiTomaso, J.M., Dyer, W.E., Edwards, M.T., Ehr, R.J., Frank, J.R., Hickman, M.V., Hill, E.R., Isensee, A.R., Koskinen, W.C., McAvoy, W.J., Mitich, L.W., Ratliff, R.L., and Sterling, T.M. 1994. Herbicide Handbook. Seventh Edition. Weed Sci. Soc. Am., Champaign, IL. 352p.
- Apajalahti, J. H. A., and Salkinoja-Salonen, M. S. 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. 169:5125-5130.
- Ashton, M. F., and Monaco, T. J. 1991. Weed Science: Principles and Practices, Third Edition, John Wiley and Sons, Inc., New York, USA., 466p.
- Aust, S. D. 1990. Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbiol. Ecol. 20:197-209.
- Banker, B. C., Kludze, H. K., Alford, D. P., DeLaune, R. D., and Lindau, C.W. 1995. Methane sources and sinks in paddy rice soils: relationship to emissions. Agriculture, Ecosystems & Environment. 53:243-251.
- Bollich, P. K., Groth, D. E., and Sanders, D. E. 1996. Influence of thiobencarb, plant residue, and dechlorinating fungus on rice. (Abstract). In: page 204, Proceedings of Twenty-Six Rice Technical Working Group, San Antonio, Texas:Feb. 25-28, 1996.
- Bouchard, B., Beaurdet, R., Villemur, R., McSween, G., Lepine, F., and Bisailon, J.-G. 1996. Isolation and characterization of *Desulfotobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol. Intern. J. System. Bacteriol. 46:1010-1015.
- Braverman, M. P., Locascio, S.J., Dusky, J. A., and Hornsby, A.G. 1990. Mobility and bioactivity of thiobencarb. Weed Sci. 38:607-614.
- Casida, J. E. Gary, R. A., and Tilles, H. 1974. Thiocarbamate sulfoxides: potent, selective, and biodegradable herbicides. Science 184:573-574.
- Cheah, U. B., and Crosby, D. G. 1995. Degradation of thiobencarb in anaerobic soils. MARDI Research Journal 23:165-169.
- Chen, C., Groth, D. E., Huffstetler, N. M., Morris, H. F., and Rush, M. C., 2000a. Microbes associated with dechlorination of thiobencarb in a specific South Louisiana rice-field soil. (Abstract). In: page 72, Proceedings of Twenty-Eight Rice Technical Working Group, Biloxi, Mississippi: February 27 – March 1, 2000.

- Chen, C., Groth, D. E., Shao, Q. M., and Rush, M. C. 2000b.** Phytotoxicity of thiobencarb and its dechlorinated product to rice. (Abstract). In: page 79-80 Proceedings of Twenty-Eight Rice Technical Working Group, Biloxi, Mississippi: February 27 – March 1, 2000.
- Chen, S-J., Hsu, E-L., and Chen, Y-L. 1982.** Fate of herbicide benthocarb (thiobencarb) in a rice paddy model ecosystem. *J. Pesticide Sci.* 7:335-340.
- Christiansen, N., and Ahring, B. K. 1996.** *Desulfitobacterium hafniense* sp. nov., an anaerobic, reductively dechlorinating bacterium. *Intern. J. System. Bacteriol.* 46:442-448.
- Cole, J. R., Cascarelli, A. L., Mohn, W. W., and Tiedje, J. M. 1994.** Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol. *Appl. Environ. Microbiol.* 60:3536-3542.
- Connell, W.E., and Patrick, W.H. Jr. 1968.** Sulfate reduction in soil: Effects of redox potential and pH. *Science.* 159:86-87.
- Conrad, R. 1996.** Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). *Microbiol. Rev.* 60:609-640.
- Crooks, G. P., and Copley, S. D. 1993.** A surprising effect of leaving group on the nucleophilic aromatic substitution reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase. *J. Am. Chem. Soc.* 115: 6422-6423.
- DeWeerd, K. A., Concannon, F., and Sufita, J. M. 1991.** Relationship between hydrogen consumption, dehalogenation, and the reduction of sulfur oxyanions by *Desulfomonile tiedjei*. *Appl. Environ. Microbiol.* 57:1929-1934.
- DeWeerd, K. A., Mandelco, L., Tanner, R., Woese, C. R., and Sufita, J. M. 1990.** *Desulfomonile tiedjei* gen. nov., and sp. nov., a novel anaerobic, dehalogenating, sulfate reducing bacterium. *Arch. Microbiol.* 154:23-30.
- Dolfing, J., and Harrison, B. K. 1992.** Gibbs free energy formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments. *Environ. Sci. Technol.* 26:2213-2218.
- Enzien, M.V., Picardal, F., Hazen, T. C., Arnold, R. G., and Fliermans, C. B. 1994.** Reductive dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment column. *Appl. Envir. Microbiol.* 60: 2200-2204.
- EPA 1997.** Reregistration eligibility decision- thiobencarb. List B Case 2665. US Environmental Protection Agency, 215p.
- Fetzner, S. 1998.** Bacterial dehalogenation. *Appl. Microbiol. Biotechnol.* 50:33-657.

Fetzner, S., and Lingens, F. 1994. Bacterial dehalogenases: Biochemistry, genetics, and biotechnological applications. *Microbiol. Rev.* 58:641-685.

Fantroussi, S.E., Naveau, H., and Agathos, S. N. 1998. Anaerobic dechlorinating bacteria. *Biotechnol. Prog.* 14:167-188.

Gambrell, R.P., and Patrick, W.H. Jr. 1978. Chemical and microbiological properties of anaerobic soils and sediments. In D.D. Hook and R.M.M. Crawford (eds.), *Plant Life in Aerobic Environments*. Ann Arbor Sci. Publ., Ann Arbor, MI, pp. 375-423.

Gantzer, C. J., and Wackett, L.P. 1991. Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* 25:715-722.

Gerritse, J., Renard, V., Pedro Gomes, T.M., Lawson, P. A., Collins, M. D., and Gottschal, J. C. 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that can grow by reductive dechlorination of tetrachloroethene or ortho-chlorinated phenols. *Arch. Microbiol.* 165:132-140.

Groth, D. E., and Sanders, D. E. 1996. Etiology and control of the delayed phytotoxicity syndrome (Abstract). In pages 203-204, *Proceedings of the Twenty-Six Rice Technical Working Group*, San Antonio, Texas:Feb. 25-28, 1996.

Groth, D. E. , Sanders, D. E., and Rich, G. 1999. Delayed phytotoxicity syndrome of rice. *Louisiana Agriculture* 42:13-14.

Hada, M., Takino, M. Yamagami, T; Daishima, S., and Yamaguchi, K. 2000. Trace analysis of pesticide residues in water by high-speed narrow-bore capillary gas chromatography-mass spectrometry with programmable temperature vaporizer. *J. Chromatography-A.* 874:81-90.

Holliger, C., and Schumacher, W. 1994. Reductive dehalogenation as a respiratory process. *Antonie van Leeuwenhoek.* 66:247-270.

Holliger, C. Wohlfarth, G., and Diekert, G. 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiology Reviews* 22:383-398.

Ishikawa, K. 1980. Studies on the fate and behavior of benthocarb herbicide in biota and the environment. *J. Pesticide Sci.* 5:287-293.

Ishikawa, K., Nakamura, Y., and Kuwatsuka, S. 1976. Degradation of benthocarb herbicide in soil. *J. Pesticide Sci.* 1:49-57.

Ishikawa, K., Nakamura, Y., and Kuwatsuka, S. 1977. Volatization of benthocarb herbicide from aqueous solution and soil. *J. Pesticide Sci.* 2:127-134.

- Ishikawa, K., Shinohara, R., Yagi, A., Shigematsu, S., and Kimura, L. 1980.** Identification of S-benzyl-N, N-diethylthiocarbamate in paddy field soil applied with thiobencarb herbicide. *J. Pesticide Sci.* 5:107-109.
- Khalid, R.A., Patrick, W.H., Jr., and DeLaune, R.D. 1977.** Phosphorus sorption characteristics of flooded soils. *Soil Sci. Soc. Am. J.* 41:305-310.
- Löffler, F. E., Ritalahti, K. M., and Tiedje, J. M. 1997.** Dechlorination of chloroethenes is inhibited by 2-Bromoethanesulfate in the absence of methanogens. *Appl. Environ. Microbiol.* 63:4982-4985.
- Ludemann, H., Arth, I., and Liesack, W. 2000.** Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* 66:754-762.
- Mabury, S. A., Cox, J. S., and Crosby, D. G. 1996.** Environmental fate of rice pesticides in California. *Rev. Environ. Contam. Toxicol.* 147:71-117.
- Mackiewicz, M., and Wiegel, J. 1998.** Comparison of energy and growth yields for *Desulfotobacterium dehalogenans* during utilization of chlorophenol and various traditional electron acceptors. *Appl. Environ. Microbiol.* 64:352-355.
- Madsen, T., and Licht, D. 1992.** Isolation and characterization of an anaerobic chlorophenol-transforming bacterium. *Appl. Environ. Microbiol.* 58:2874-2878.
- Manun, A. A., and Shimizu, M. 1978a.** Studies on a herbicide, benthocarb. I. Effects of benthocarb on gross morphology of barnyardgrass *Echinochloa crusgalli*. *Jpn. J. Crop Sci.* 47:563-572.
- Manun, A. A., and Shimizu, M. 1978b.** Studies on a herbicide, benthocarb. II. Effects of different amounts of benthocarb treated at different growth stages on the morphogenesis of barnyardgrass *Echinochloa crusgalli*. *Jpn. J. Crop Sci.* 47:573-580.
- Manun, A. A., and Shimizu, M. 1979a.** Studies on a herbicide, benthocarb. III. Morphological and histological effects of benthocarb in controlling weeds, specially the barnyardgrass *Echinochloa crusgalli* on upland rice. *Jpn. J. Crop Sci.* 48:139-147.
- Manun, A. A., and Shimizu, M. 1979b.** Studies on a herbicide, benthocarb. IV. Histological effects of benthocarb on barnyardgrass [*Echinochloa crusgalli* var *oryzichola*]. *Jpn J Crop Science* 48:393-402.
- Masscheleyn, P.H., DeLaune, R.D., and Patrick, W.H., Jr. 1993.** Methane and nitrous oxide emissions from laboratory measurements of rice soil suspension: Effect of soil oxidation- reduction status. *Chemosphere* 26:251-260.

- Mohn, W. W., and Tiedje, J. M. 1991.** Evidence for chemiosmotic coupling of reductive dechlorination and ATP synthesis in *Desulfomonile tiedjei*. Arch. Microbiol. 157:1-6.
- Mohn, W. W., and Tiedje, J. M. 1992.** Microbial reductive dehalogenation. Microbiol. Rev. 5:482-507.
- Moon, Y. H., and Kuwastuka, S. 1984.** Properties and conditions of soils causing the dechlorination of the herbicide benthocarb (thiobencarb) in flooded soils. J. Pesticide Sci. 9:745-754.
- Moon, Y. H., and Kuwatsuka, S. 1985a.** Microbial aspects of dechlorination of the herbicide benthocarb (thiobencarb) in soil. J. Pesticide Sci. 10:513-521.
- Moon, Y. H., and Kuwatsuka, S. 1985b.** Factors influencing microbial dechlorination of benthocarb (thiobencarb) in the soil suspension. J. Pesticide Sci. 10:523-528.
- Moon, Y. H., and Kuwatsuka, S. 1985c.** Characterization of microbes causing dechlorination of benthocarb (thiobencarb) in diluted soil suspension. J. Pesticide Sci. 10:541-548.
- Moon, Y. H., and Kuwatsuka, S. 1987a.** Nutritional conditions for microorganisms to dechlorinate benthocarb (thiobencarb). J. Pesticide Sci. 12:3-10.
- Moon, Y. H., and Kuwatsuka, S. 1987b.** Population changes of benthocarb (thiobencarb)- dechlorinating microorganism in soil. J. Pesticide Sci. 12:11-16.
- Nakamura, Y., Ishikawa, K., and Kuwatsuka, S. 1974.** Uptake and translocation of benthocarb herbicide by plants. Agric Biol. Chem. 38:1129-1135.
- Nakamura, Y., Ishikawa, K., and Kuwatsuka, S. 1977.** Degradation of benthocarb in soils as affected by soil conditions. J. Pesticide Sci. 2:7-16.
- Ni, S., Fredrickson, J. K., and Xun, L. 1995.** Purification and characterization of a novel 3-chlorobenzoate reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. J. Bacteriol. 177:5135-5139.
- Patrick, W.H., Jr., and DeLaune, R.D. 1972.** Characterization of the oxidized and reduced zones in flooded soil. Soil Sci. Soc. Am. Proc. 36:573-576.
- Patrick, W.H., Jr., Gambrell, R.P., and Faulkner, S.P. 1996.** Redox measures of soils. In: pages 1255-1273 Chapter 42. Methods of Soil Analysis. Part 3. Chemical Methods-SSSA Book Series No.5.

- Patrick, W.H., Jr., and Henderson, R.E. 1981.** Reduction and reoxidation cycles of manganese and iron in flooded soil and in water solution. *Soil Sci. Soc. Am. J.* 45:855-859.
- Patrick, W.H., Jr., and Jugsujinda, A.. 1992.** Sequential reduction and oxidation of inorganic nitrogen, manganese and iron in flooded soil. *Soil Sci. Soc. Am. J.* 56:1071-1073.
- Patrick, W. H., Jr., Mikkelsen, D. S. and Wells, B. R. 1985.** Plant nutrient behavior in flooded soil. Pages 197-228. In: *Fertilizer Technology and Use* (3rd Edition). Soil Science Society of America.
- Prakash, T. R., Swamy, P. M., and Prasad, D. D. K. 1990.** Selective inhibition of porphyrin biosynthesis by benthocarb (Saturn) in rice and barnyard grass seedlings. *Pestic. Biochem. Physiol.* 36:29-34.
- Quensen, J. F., III, Boyd, S. A., and Tiedje, J.M. 1990.** Dechlorination of four commercial polychlorinated biphenyl mixture (Aroclors) by anaerobic microorganisms from sediments. *App. Environ. Microbiol.* 56:2360-2369.
- Reddy, K.R., D'Angelo, E., Lindau, C., and Patrick, W.H., Jr. 1990.** Urea losses in flooded soils with established oxidized and reduced soil layers. *Biol. Fertil. Soils* 9:283-287.
- Reddy, K. R., Patrick, W. H., Jr., and Lindau, C. W. 1987.** Redox processes and nitrogen transformations in flooded soils. In page 729-737 *Transactions - 13th Congress of Int'l Society of Soil Science, Hamburg, Germany, Aug. 13-Aug. 20, 1986. Vol. 6*
- Reddy, K. R., Rao, P. S. C., and Patrick, W. H., Jr. 1980.** Factors influencing the oxygen consumption rates in flooded soils. *Soil Sci. Soc. Am. J.* 44:741-744.
- Reddy, G. V. B., Gelpke, M. D. S., and Gold, M. H. 1998.** Degradation of 2,4,6-trichlorophenol by *Phanerochete chrysosporium*: Involvement of reductive dechlorination. *J. Bacteriol.* 180:5159-5164.
- Ross, L. J., and Sava, R. J. 1986.** Fate of thiobencarb and molinate in rice fields. *J. Environ. Qual.* 15 :220-225.
- Sanders, D. E., Groth, D. E., and Strahan, R. E. 1996.** Rice injury and injury prevention resulting from interactions between soilborne fungi and selected rice herbicides and fungicides. *Proc. South. Weed Sci. Soc.* 49:44.
- Sanford, R. A., Cole, J. R., Loffler, F. E., and Tiedje, J. M. 1996.** Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. *Appl. Environ. Microbiol.* 62:3800-3808.

Schabenberger, O., Tharp, B. E., Kells, J. J., and Penner, D. 1999. Statistical tests for hormesis and effective dosages in herbicide dose response. *Agronomy J.* 91:713-721.

Schenzle, A., Lenke, H., Spain, J. C., and Knackmuss, H-J. 1999. Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134. *Appl. Envir. Microbiol.* 1999 65: 2317-2323.

Scholz-Muramatsu, H., Neumann, A., Mebmer, M., Moore, E., and Dickert, G. 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strict anaerobic bacterium. *Arch. Microbiol.* 163:48-56.

Seefeldt, S. S., Jensen, J. E., and Fuerst, E. P. 1995. Log-logistic analysis of herbicide dose-response relationships. *Weed Technol.* 9:218-227.

Stark, J. M. 1996. Shaker speeds for aerobic soil slurry incubations. *Comm. Soil Sci. Plant Anal.* 27:2625-2631.

Streibig, J. C., Rudermo, M., and Jensen, J. E. 1993. Dose-response curves and statistical models. P.30-55. In: J. C. Streibig and P. Kudsk, eds. *Herbicide Bioassays*. CRC Press, Boca Raton, FL.

Sufliata, J. M., Horowitz, A., Shelton, D. R., and Tiedje, J. M. 1982. Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. *Science* 218:1115-1116.

Sun, B., Cole, J., Sanford, R. A., and Tiedje, J. M. 2000. Isolation and characterization of *Desulfovibrio dechloracetivorans* sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol. *Appl. Environ. Microbiol.* 66:2408-2413.

Tatsuyama, K., Yamamoto, H., and Egawa, H. 1981. Bioassay of dechlorination of benthiocarb (thiobencarb) herbicide in flooded soil using germinated grains of rice plants. *J. Pesticide Sci.* 6:193-199.

Tjeerdema, R. S., and Crosby, D. G. 2000. The microbial degradation of pesticide important to rice culture-2000. <http://www.syix.com/rrb/00rpt/Microbial.htm>

Tsuchiya, T., and Yamaha, T. 1983. Reductive dechlorination of 1,2,4-trichlorobenzenes on incubation with intestinal contents of rats. *Agri. Biol. Chem.* 47:1163-1165.

Tsuchiya, T., and Yamaha, T. 1984. Reductive dechlorination of 1,2,4-trichlorobenzenes by *Staphylococcus epidermidis* isolated from intestinal contents of rats. Agri. Biol. Chem. 48:1545-1550.

Uno, Y., Kinoshita, T., and Takahashi, M. 1977. Differential responses to herbicides found between Japonica and Indica varieties of rice. Jpn. J. Breed. 27:226.

Utkin, I., Woese, C., and Wiegel, J. 1994. Isolation and characterization of *Desulfotobacterium dehalogenans* gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds. Intern. J. Syst. Bacteriol. 44:612-619.

van den Tweel, W. J. J., Kok, J. B., and de Bont, J. A. M. 1987. Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by *Alcaligenes denitrificans* NTB-1. Appl. Environ. Microbiol. 53 :810-815.

Wang, Z. P., Delaune, R. D., Masscheleyn, P. H., and Patrick, W. H., Jr. 1993. Soil redox and pH effects on methane production in a flooded rice soil. Soil Sci. Am. J. 57:382-385.

Yadav, J. S., Quensen, J. F., Tiedje, J. M., and Reddy, C. A. 1995. Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by Congener-specific analysis. Appl. Environ. Microbiol. 61:2560-2565.

Yamada, T. 1982. Eliminating method of elemental sulfur for gas chromatographic determination of benthioncarb (thiobencarb) and dechlorinated benthioncarb in paddy soil. J. Pesticide Sci. 7:23-30.

Vita

Chiliang Chen, was born on October 1, 1965, in Hexian County, Guangxi Province, People's Republic of China. He is the oldest son among five sons in the family of Jianhong Chen and Juzhen Li. Growing up in the countryside, he went to a nearby elementary school to be educated in elementary and middle school. He was admitted to Hexian County High School from 1979 to 1982. From 1982 to 1986, he studied in the Department of Plant Protection of Guangxi Agricultural College (renamed Guangxi University) in Nanning, Guangxi Province. After graduating from college, he was assigned to the Institute of Plant Protection in Guangxi Academy of Agricultural Sciences in the same city. After spending nine years on research focused on the rice blast disease, he realized he needed more professional training. In the fall of 1995, he began graduate studies in the Department of Plant Pathology and Crop Physiology, Louisiana State University. He first studied in the laboratory of Dr. C. A. Clark and received a master's degree in Plant Health (Plant Pathology) in December 1997. He then began his doctoral program in the same department with Dr. D. E. Groth and Dr. M. C. Rush as co-advisors. He married Canxia Qin in August 1996. Their son, Tilden Chen, was born in 1998 in Baton Rouge, Louisiana. Chiliang is a member of the American Phytopathological Society and the American Society for Microbiology. He is now a candidate for the degree of Doctor of Philosophy.